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ENDOCRINOLOGY

VOLUME 45 JULY, 1949 NUMBER 1

EFFECT OF CARBON TETRACHLORIDE-FEEDING ON ESTROGEN EXCRETION IN THE NORMAL FEMALE GUINEA PIG[1]

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LOS ANGELES, CALIF.

IN ORDER to confirm and extend previous investigations in which increases in urinary estrogens were found in rats after partial hepatectomy (Schiller and Pincus, 1944), and in humans who had chronic liver cirrhosis accompanied by gynecomastia (Glass, Edmondson and Soll, 1940, 1944) or acute hepatitis (Gilder and Hoagland, 1946), the total estrogen excretion has been studied in normal female guinea pigs before, during and after carbon tetrachloride feeding.

EXPERIMENTAL

Materials and Methods: Six female guinea pigs weighing an average of 815 g. at the beginning of the experiment were used. Urine was collected irregularly over a period of 164 days before the CCl_4 treatment was begun, over 141 days during CCl_4 feeding, over a 45-day period after cessation of CCl_4 feeding and over a 16-day period during which CCl_4 feeding was resumed. Each guinea pig was fed twice weekly with 0.4 ml. of a CCl_4-Wesson oil mixture in which the CCl_4 content was varied from 0.02 to 0.08 ml. per dose. These dosage levels for CCl_4 were sublethal as compared with 100 per cent fatality obtained within seven days after one administration of 0.2 ml. CCl_4.

Each urine sample was collected for 72 hr. periods over 25 ml. 12N HCl. The urine was filtered, hydrolyzed for 15 min. with 0.15 vol. 12N HCl and extracted with diethyl-ether. The ether extract was washed with H_2O and Na_2CO_3 and the total phenolic steroids were extracted with 2N NaOH, acidified and re-extracted with ether according to the method of Pincus (1945).

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[2] Ayerst, McKenna and Harrison, Ltd., Fellow.

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TABLE 1. AVERAGE VOLUME OF URINE AND AMOUNT OF ESTROGEN EXCRETED BY FEMALE GUINEA PIGS BEFORE, DURING, AND AFTER CCl_4 FEEDING

Sample number	Urine period start	Collection (days)* finish	Average volume urine per G.P. per day (ml.)	Number of doses of CCl_4	Total vol. CCl_4 (ml.)	Ave. mcg. estrogen L. urine‡	Ave. mcg. estrogen G.P./day‡
3	−143	−139	21.0			2.34	.0491
6	−107	−104	33.1			1.00	.0331
12	− 38	− 35	22.2			1.50	.0332
14	− 3	0	25.5			0.95	.0243
Average Normal			21.0±6.6			1.45±0.64	.0349± .0103
15	4	7	23.6	2	0.04	6.11	.144
16	11	14	23.3	4	0.08	10.00	.238
18	25	28	19.4	8	0.16	52.0	1.009
19	32	35	28.8	10	0.20	319.00	9.13
20	47	50	27.2	12	0.24	495.00	13.45
21	67	70	29.4	19	0.44	120.00	3.53
22	74	77	31.6	21	0.51	50.00	1.60
23	88	92	34.4	25	0.67	18.9	.649
24	102	106	28.8	29	0.86	4.61	.133
25	132	135	26.9	33	1.09	9.13	.247
26	141	145	19.5	36	1.33	6.33	.123
Average			26.9±4.5				
27	188	193	15.4			2.03	.0314
28	193	200	20.0			1.56	.0311
29	204	209	18.6			2.57	.023
Average			18.2±1.9				
30	209	214	15.3	1†	0.08	4.38	.0670
31	214	221	15.7	2†	0.16	12.5	.270
32	221	225	12.6	3†	0.24	15.3	.330
Average			17.6±2.7				

* 0 = day first dose of CCl_4 was given, and a negative number = days before CCl_4 feeding was begun.
† A second series of CCl_4 feedings.
‡ Estrogen expressed as mcg. estrone equivalents.

The ether was evaporated and the residue stored in a vacuum desiccator. For bioassay of the total estrogenic activity the residue was dissolved in sesame oil and administered intravaginally according to the method of Lyons and Templeton, 1935, on spayed female rats which had been standardized previously (Pugsley and Morrell, 1943). Smears were read according to Kahnt and Doisy (1928). All rats were primed with 0.15 mcg. estrone every 14 days and assays were carried out during the intervening weeks.[3]

Results: The results of the assays of the urine extracts, shown in table 1 and figure 1, have been correlated with the dose and time at which CCl_4 was administered, and with the volume of urine excreted. The estrogen excretion (expressed as mcg. equivalents of estrone) has been plotted as concentration per liter of urine and as amount per guinea pig per day, against time in days. The assays were calculated by the probit method and it was found that the average deviation for the method was 9.8 per cent. A standard deviation of 43.4 per cent was obtained for the estrogen concentration in the urine of normal animals while the standard deviation of the amount of estrogen excreted per animal per day was 26.8 per cent. This indicated that in normal animals total daily excretion was more constant than the urinary concentration.

[3] Crystalline estrone was supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J., and by Eli Lilly Co., Indianapolis, Ind.

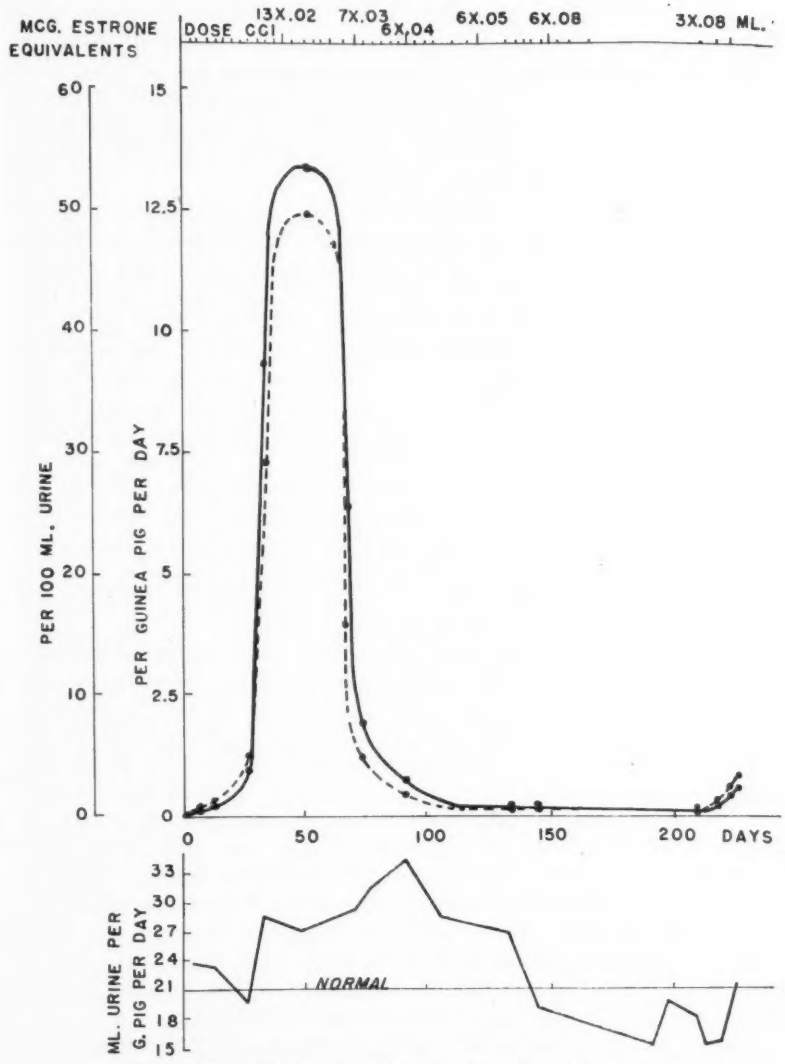

FIG. 1. Effect of CCl_4 Feeding on Estrogen Excretion and on Urine Volume of Female Guinea Pigs.*

* Estrogen excretion per guinea pig per day shown by solid line of upper curve; estrogen concentration per 100 ml. urine shown by broken line of upper curve. Figures arranged horizontal at top indicate size and number of doses of CCL_4 as well as time of administration.

Lower curve shows change in average volume of urine excreted per guinea per day during course of the experiment. Horizontal line in this graph indicates average, normal urine volume (approximately 21 ml. 1 g. pig/day).

The data in table 1 and figure 1 show significant changes in urine volume as a result of CCl_4 feeding. The mean volume of urine excreted by normal animals was 21.0 ml. per guinea pig per day, with a standard deviation of 6.6 ml.; the mean volume during 141 days of CCl_4 feeding was 26.9 ± 4.5 ml.; at the end of the recovery period, the mean volume was 18.2 ± 1.9 ml.; a further decrease to 17.6 ± 2.7 ml. occurred when CCl_4 feeding was resumed.

It will be noted that when CCl_4 was fed to normal guinea pigs the excretion of endogenous estrogenic substances showed three distinct stages: (1) The estrogen excretion increased during the first 50 days, at first slowly and then rapidly, represented by a sigmoid curve. This was accompanied by an initial fall in the volume of urine which returned to a level significantly above normal during the period of strikingly elevated estrogen excretion. The excretion of estrogen per day increased at a faster rate than the estrogen concentration in the urine until the peak was reached (except for a lag during the first 25 days). The estrogen excretion at the peak of the curve was approximately 350 times the normal value. (2) The estrogen excretion decreased during the second 50 days, at first rapidly and then slowly, also represented by a sigmoid curve. The total estrogen excretion decreased more slowly than the concentration until the 88th day. (3) The estrogen excretion was relatively constant at a level 3.7 times normal throughout the remaining 50 days of CCl_4 feeding.

It will be noted that the slope of the daily excretion curve between any two points, compared to the slope of the concentration curve between corresponding points, always was less in the presence of a decrease in urine volume and greater in the presence of an increase in volume except between days 88 and 141 (see fig. 1). At the end of the period during which no CCl_4 was fed (to permit recovery), both the concentration and total output of estrogen decreased, so that they fell within the normal range. The greater drop of the total estrogen excretion again was coincident with further decrease in urine volume. On administering a second series of CCl_4 feedings, it was interesting to note that, in spite of pre-existent liver damage, the effect should so closely duplicate that of the first series, both in the fall of urine volume and the increase in estrogen excretion, at least for the short period covered by this second series of CCl_4 feedings.

Autopsy of two animals at the end of the recovery period showed the vaginae to be closed and previously noted edema of the subcutaneous tissues had disappeared almost entirely. No adhesions or fibroids were found in the abdominal cavity. The livers showed advanced secondary cirrhosis, with no signs of recovery. They were dark red with a "lemon rind" appearance. The ovaries were enlarged and numerous follicles were present. The adrenals were hemorrhagic and only slightly, if at all, enlarged.

DISCUSSION

Estrogen Excretion in Normal Female Guinea Pigs

Emmens (1943) determined estrogen excretion in the normal female guinea pig and found 377 ± 80 "effective intravaginal mouse doses" excreted per guinea pig per five days, with a range from 90 to 600 effective doses. He defined one effective intravaginal mouse dose as 0.0029 mcg. of estrone. This is equal to $.0218 \pm .0046$ mcg. per guinea pig per day with a range from .0052 to .0348 mcg. The average excretion of $.0349 \pm .0103$ mcg. per guinea pig per day (range from .0234 to. 0491 mcg.) reported here compared with the values obtained by Emmens, shows that our results were higher but the average falls within the upper limits of Emmens' range. His average value is only slightly below the range reported by us. This discrepancy may be due in part to a difference in age, strain or nutritional state of the two groups of animals or to a difference in the method of extraction, since he used benzene which is less efficient than ether. In addition, the use of the mouse instead of the rat as the assay animal has been shown to give lower values and less accurate results (Hain and Robson, 1936, and Curtis, Witt and Knudsen 1944).

Beerstecher (1942) found that the average volume of urine excreted by virgin female rabbits varied inversely with the concentration of estrogen. He reported an average of 30 ml. per rabbit per day from August to May when the estrogen concentration was 1.0 mcg. per liter, and 20 ml. per day from May to August when the concentration was 2.0 mcg. per liter.[4] In our studies with the normal female guinea pigs the inverse ratio of estrogen concentration to volume of urine excreted was observed also and was more constant than either the direct or inverse ratio of total estrogen per day to urine volume. Thus, the product (concentration $\times$ volume), which is the total estrogen excretion per day, tends to approach a constant value.

Effect of CCl_4 Administration on Estrogen Excretion

(1) Estrogen excretion during first 50 days: The increased excretion of estrogen during the first 50 days of CCl_4 feeding agrees with the findings of Talbot (1939) and of Pincus and Martin (1940) who reported increased activity of endogenous and exogenous estrogens after CCl_4 administration to rats. Talbot found the uterine weight of rats fed CCl_4 was increased above normal while the weight of uteri of ovariectomized rats did not increase with CCl_4 administration. Pincus and Martin found a given dose of estrogen was 80 per cent more

[4] It is surprising that the volume of urine excreted per day by the rabbits reported by Beerstecher is only slightly more than the volumes excreted per day by our guinea pigs.

effective in causing vaginal estrus after CCl_4 administration to rats than in nontreated animals. It should be pointed out, however, that in both experiments CCl_4 was administered for only short periods of time and then only slightly sublethal doses were used, and that the effects of CCl_4 on estrogen excretion were not determined.

It has been demonstrated also that any impairment of normal liver function results in an enhanced effect of both endogenous and exogenous estrogens. Such impairment has been obtained by: 1) vitamin B complex deficiency diets in rats by Biskind and Biskind (1941, 1942), Biskind and Selesnyak (1942), Segaloff and Segaloff (1944), in mice and rats by Szego and Barnes (1943), and Shipley and Gyorgy (1944); 2) inanition in rats by Shipley and Gyorgy (1944) and Drill and Pfeiffer (1946); 3) partial hepatectomy in rats by Schiller and Pincus (1944) and Roberts and Szego (1947); 4) chronic liver cirrhosis with gynecosmatia in men by Glass, Edmondson and Soll (1940, 1944); and 5) acute infectious hepatitis in men by Gilder and Hoagland (1946).

The reduction of estrogenic effect has been localized to the liver *in vivo* by reports of a greater effect of systemic as compared to portal introduction of both endogenous and exogenous estrogens (Evans and Burr, 1922; Golden and Sevringhaus, 1938; Biskind and Mark 1939; G. R. Biskind, 1941a, 1942; Segaloff and Nelson, 1941; Segaloff, 1943; Engel, 1944). Further evidence that the liver is the site of estrogen inactivation has been obtained by the marked decrease in estrogenic activity 1) after perfusion of estrogen through the liver of dogs (Israel and Meranze, 1937) and rats (Schiller, 1945), and 2) after *in vitro* incubation of estrogen with liver tissue of dogs (Silberstein, Molnar and Engel, 1933 and Zondek, 1934), rats and rabbits (Heller, Heller and Sevringhaus, 1939, Heller, 1940) and guinea pigs in different sexual states (Engel, 1941).

Localization of estrogen inactivating ability to the hepatic cells rather than the Kupfer cells was obtained by Zondek and Sklow (1941) who inhibited the reticulo endothelial system with colloidal copper. That the inactivation is enzymatic is indicated by the work of Zondek (1934) who found that liver tissue lost its inactivating ability after heating, by Heller (1940) who reported inhibition of inactivation by NaCN and by Engel (1945) who obtained inactivation with an aqueous extract of beef liver, the active principle of which could be concentrated by alcoholic precipitation. However, Cantarow, Paschkis, Rakoff and Hansen (1943) were not able to find any difference in ability to inactivate estradiol between normal rat liver tissue and liver from animals fed with CCl_4. Their findings suggest that some mechanism other than enzyme inactivation may be responsible for the enhanced estrogenic activity *in vivo* following administration of CCl_4.

In view of the evidence cited above, it seems probable that the in-

creased estrogenic activity obtained in the urine when CCl_4 is fed to normal female guinea pigs may be due to an impairment of the ability of the liver to inactivate these hormones. Two stages of increased estrogen excretion are present, the first at a slow rate accompanied by a decreased urine volume (day *0* to day *14*), the second a sharp increase accompanied by larger urine volume (day *14* to day *50*). It is likely that the ability of the liver to convert the more active estrogens to estriol is lost first, since conversion of estrone to estriol is reduced in hepatectomized rats (Schiller and Pincus 1944). Following this, loss of interconversion between estrone and estradiol presumably would leave only the most active estrogen, estradiol, since this is the compound reportedly formed by the ovary. Since CCl_4 is known to be a nonspecific toxic agent and since other tissues than the liver are capable of this conversion in the normal animal (Heller, 1940) it is possible that the magnitude of the increased estrogen excretion obtained in our experiments may be due to an impairment of this mechanism, not only in the liver, but in other tissues as well. The marked changes in the average volume of urine excretion during the course of this experiment suggests a change in kidney function that may be due, directly or indirectly, to the cytotoxic action of CCl_4.

(2) Estrogen excretion from day 50 to day 145: The decrease in estrogen excretion, following the initial increase, has not been reported previously to our knowledge. Moreover, a peak of estrogen excretion as great as 350 times the normal level also has not been reported previously with endogenous estrogens as the only source. The use of more severe experimental procedures, carried out for a relatively short time, may be responsible for the failure of previous investigators to obtain these results. In the studies using hepatotoxic agents only a few relatively massive doses were administered. For example Pincus and Martin (1940) used 0.1 ml. CCl_4 three times a week in rats while we used only 0.02 to .08 ml. twice a week in guinea pigs. Thus, the dose/body weight ratio was much smaller in our experiments. In studying the effect of vitamin B complex deficient diets and of inanition (described above) the experiments extended over a total of only 35 to 60 days with no evidence of enhanced estrogen activity during the first 30 days, while CCl_4 fed animals showed an immediate increase in estrogen excretion but the peak, followed by decrease in excretion, was not reached until 50 days after such treatment. It is possible that if the dietary experiments had been continued for a longer time a peak of activity, followed by regression, might have been observed. The absence of histologic change in the liver during the first few weeks with certain types of dietary deficiency as compared to severe hepatic damage, even within the first few days of CCl_4 feeding, indicates the former to be a less severe process. When hepatectomy is used to damage the liver, the acute decrease in functioning liver

tissue, followed by rapid regeneration, may preclude the production of secondary changes in other organs that are found when chronic liver damage is maintained by repeated assaults of sublethal doses of CCl_4.

The marked decrease in estrogen excretion observed between day 50 and day 100 suggests several possibilities 1) that estrogens are not being produced because of damage by CCl_4 to the pituitary or the ovaries, 2) that activation of the estrogens by the liver does not occur (Szego and Roberts 1946), or 3) that inactivation of estrogens is resumed either by the liver or by some other organ.

(3) Recovery from day 145 to day 209: When the effects of extended CCl_4 administration are allowed to regress for 45 days, both the total daily excretion and urinary concentration of estrogen return to the normal range (although urine volumes were significantly lower than normal). Advanced secondary cirrhosis was present in the livers and showed no signs of recovery. This observation and those of Cantarow, *et al.* (1943) suggest that normal liver morphology need not be necessary for estrogen inactivation and that physiological repair (at least a return of the function of estrogen inactivation) may precede morphological repair in the liver.

Effect of CCl_4 Feeding After Recovery (day 209 to 225)

When CCl_4 feeding was resumed after a 45-day period of rest, an increased estrogen excretion and decreased urine volume occurred as it did following the initial CCl_4 feedings. This duplication of the original findings suggests that the function of inactivation returns toward normal soon after cessation of treatment with the toxic agent.

Probably no one single hypothesis is adequate to explain the effects of CCl_4 feeding on estrogen excretion, but rather, they are due to a series of factors produced by, 1) direct cytotoxicity of CCl_4, and 2) the indirect effect of functional impairment by CCl_4 on interdependent processes.

SUMMARY

By the use of the intravaginal assay method it is found the normal female guinea pig excretes $0.035 \pm .010$ mcg. estrone equivalents per day with a range from 0.023 to 0.049. When CCl_4 is fed, by the method and dosage described, the following changes in estrogen excretion occur (expressed as mcg. estrone equivalents):

(1) During the first 50 days the level of estrogen excretion increase to a peak 350 times normal.
(2) During the second 50 days, the excretion level decreases, a phenomenon not reported previously.
(3) During the third 50 days, a fairly constant level of estrogen excretion is maintained at approximately 3.7 times normal.

When the effects of extended CCl_4 feeding are allowed to regress for 45 days the levels of estrogen excretion return to normal. Resump-

tion of CCl_4 administration results in an increase in estrogen excretion similar to that following initial CCl_4 feeding.

It is suggested that the sequence of effects of CCl_4 administration are 1) an impaired inactivation of estrogens resulting in increased excretion, followed by 2) either an inhibition in the production of endogenous estrogens, or, loss of activation of estrogens resulting in decreased estrogen excretion.

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THE DETERMINATION OF ADRENOCORTICAL STEROIDS IN HUMAN URINE[1]

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INTRODUCTION

VARIOUS methods for the determination of adrenocortical steroids in human urine have been proposed in recent years. Hormonally active corticosteroids have been measured by bioassay (e.g., Selye and Schenker 1938, Eggleston *et al.* 1946, Venning *et al.* 1946), but the relatively small concentrations of active material, particularly in normal human urine (Venning and Kazmin 1946, Heard 1948) has required the processing of large amounts of urine. The probability of the presence in urine of typical adrenocortical substances lacking biological activity has led to attempts at their measurement. Advantage has been taken of the fact that certain typical adreno-corticosteroids possess significant reducing activity by virtue of an α-ketol side chain at C-17 (Talbot *et al.* 1945) as well as the α, β unsaturation in ring A (Heard and Sobel 1946). In addition fairly specific determination of certain types may be had by periodic acid oxidation which generates formaldehyde from either α-ketol or glycol side chains (Lowenstein *et al.* 1946, Daughaday *et al.* 1948). In this paper we will compare the results of assay of reducing with formaldehydogenic activity.

METHODS

The urine for assay was collected from normal, healthy men and women of various ages. Two types of collection were made. The first involved three collections per 24 hours covering the periods of sleep, early morning, and rest of day (Pincus 1945). This type of collection permits the assessment of the diurnal rhythm of excretion of certain urinary steroids (Pincus *et al.* 1948a). The second consisted of three specimens from subjects in the basal state covering (a) a period (called prestress) of two hours preceding the administration of 50 mgm. equivalent of the Armour standard of pituitary adrenocorticotrophin[2] (ACTH), (b) a period (labelled stress) of one hour and fifteen

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minutes following ACTH administration, and (c) a period (labelled post-stress) of two hour following the stress collection.

The urines were extracted by the method of Heard and Sobel (1946) to yield a neutral lipid fraction. Aliquots were then made for further processing or colorimetric assay. The neutral lipid extract of certain urines was partitioned between benzene and water by the method of Talbot *et al.* (1945) to give a crude "C" fraction containing all benzene-soluble neutral lipid which is then extracted with water to give "benzene" and "water" fractions.

A separation of alcoholic lipids from the non-alcoholic constituents was effected by the method of Pincus and Pearlman (1941).

Determination of reducing lipid were made by the phosphomolybdic acid method (PRL) (Heard and Sobel 1946) and the copper reduction method (CRL) of Talbot *et al.* (1945). Formaldehydogenic lipid (FL) was determined by a 20-minute periodic acid oxidation followed by colorimetric measurement of the distilled formaldehyde generated (cf. Daughaday *et al.* 1948). All determinations are expressed as equivalents of a desoxycorticosterone standard.

RESULTS

Phosphomolybdic acid reducing lipids and formaldehydogenic lipids

In Tables 1 and 2 we present the data on diurnal urine collections from 34 men and 34 women, respectively, giving desoxycorticosterone equivalents of the neutral reducing lipids (PRL) determined by the Heard-Sobel method and of the formaldehyde generating lipids (FL). The latter method invariably gives lower values than the former. In both sets of data the tendency for individual morning and day values to exceed the sleep values is evident, as is the tendency for the men to excrete more reducing lipid, on the average, than the women.

In Tables 1 and 2 we present the mean output values (and their standard errors) and mean percentage change of the morning and day values over the sleep values. It should be pointed out that the mean percentage change is an average of the individual percentage changes which are not shown in the table. By the "t" test the data for men exhibit significantly higher absolute outputs (mgm. per 24 hours) of neutral reducing lipids in both the morning and the day values over the sleep values, while the data for the women do not. Statistically significant increase in FL is obtained in both groups for the morning increase over sleep but not for the day over sleep. When we consider the mean percentage increases of the morning and the day values over the sleep values all changes are significant for both men and women. As we have pointed out elsewhere (Pincus *et al.* 1949) taking the percentage changes tends to bring together the data for individuals having different characteristic absolute levels of output. We may therefore conclude that the data for both the men and women in this series exhibits a diurnal rhythm. Such a diurnal rhythm has been previously noted for neutral reducing lipids in men (Pincus *et al.* 1948a).

It should be pointed out that although the formaldehydogenic

TABLE 1. NEUTRAL REDUCING LIPID (PRL) AND FORMALDEHYDOGENIC LIPID (FL). URINARY OUTPUTS OF NORMAL, HEALTHY MEN

Subject No.	Age Yrs.	Mg. per 24 hours					
		PRL*			FL*		
		Sleep	Morning	Day	Sleep	Morning	Day
1	23	3.58	2.90	3.26	0.32	0.46	0.34
2	23	3.78	3.76	1.98	0.69	0.50	0.35
3	26	1.85	1.87	2.76	0.34	0.39	0.54
4	28	3.16	5.93	4.77	0.34	0.72	0.48
5	29	2.03	1.97	2.56	0.30	0.44	0.29
6	32	1.91	3.13	2.43	0.30	0.55	0.36
7	32	2.92	5.60	3.19	0.39	0.36	0.35
8	34	1.42	2.00	2.42	0.17	0.30	0.28
9	35	2.31	3.76	2.48	0.44	0.64	0.30
10	36	1.49	2.50	2.32	0.27	0.48	0.30
11	37	1.28	1.62	1.80	0.55	0.21	0.91
12	39	1.82	1.42	2.33	0.22	0.24	0.30
13	41	2.14	3.66	1.35	0.35	0.82	0.31
14	42	2.76	3.48	1.57	0.32	0.41	0.29
15	45	2.40	2.52	4.20	0.14	0.28	0.30
16	46	1.74	2.75	2.65	0.29	0.40	0.39
17	47	1.79	1.15	1.75	0.13	0.29	0.22
18	47	1.52	1.80	2.64	0.22	0.30	0.34
19	47	2.38	2.61	2.44	0.31	0.47	0.37
20	47	2.56	3.12	2.80	0.35	0.58	0.50
21	47	2.49	1.90	2.88	0.20	0.19	0.25
22	49	2.50	3.43	3.01	0.41	0.61	0.45
23	51	1.99	—	2.16	0.65	—	0.34
24	52	1.32	2.33	1.91	0.17	0.63	0.40
25	52	1.30	2.10	3.48	0.28	0.36	0.54
26	52	2.23	2.78	4.28	0.60	0.48	0.70
27	54	1.60	1.46	1.99	0.22	0.31	0.32
28	55	1.91	2.38	1.75	0.24	0.31	0.21
29	61	1.24	1.75	1.84	0.19	0.30	0.14
30	61	1.73	1.45	1.71	0.10	0.31	0.13
31	61	1.84	2.52	2.16	0.27	0.38	0.23
32	63	1.92	2.34	3.28	0.21	0.40	0.27
33	76	1.46	3.34	1.72	0.24	0.22	0.18
34	80	3.41	1.17	1.50	0.79	0.34	0.29
Means †		2.11 ± 0.115	2.62 ± 0.193	2.51 ± 0.140	0.32 ± 0.027	0.41 ±0.026	0.35 ±0.026
Mean % increase over sleep †		—	28 ±7.3	27 ±7.5	—	54 ±11.9	23 ±8.2

* Expressed as 11-desoxycorticosterone equivalent.

† Numbers underlined with a single line give a "t" value for difference from the sleep determinations @ the 2% to 5% level of confidence, those underlined with a double line give a "t" value @ the 1% level of confidence or below.

lipid shows a greater net increase in the morning in both sexes than does the neutral reducing lipid (col. 3) the percentage increases are nonetheless not significantly different for the men's data ($t = 1.87$, $p > .05$) but are for the women's data ($t = 2.38$, $p < .05$). In brief, the increase in FL observed in the morning hours are larger than the increases of PRL, but they are more variable and so are of questionable significance. That the variability of the PRL method is less than the FL method is obvious from the coefficients of variation of the data. For instance, the FL coefficients of variation for the sleep determina-

TABLE 2. NEUTRAL REDUCING LIPID (PRL) AND FORMALDEHYDOGENIC LIPID (FL). URINARY OUTPUTS OF NORMAL, HEALTHY WOMEN

Subject No.	Age Yrs.	Mg. per 24 hours					
		PRL*			FL*		
		Sleep	Morning	Day	Sleep	Morning	Day
1	22	1.70	1.70	1.34	0.46	0.43	0.15
2	22	1.47	2.16	3.79	0.19	0.46	0.47
3	23	2.38	2.26	2.35	0.23	0.43	0.35
4	26	1.44	1.94	2.12	0.82	0.71	0.68
5	26	1.31	1.21	1.45	0.33	0.47	0.36
6	29	1.54	1.95	1.60	0.31	0.23	0.62
7	31	2.24	2.60	3.17	0.39	0.38	0.18
8	31	0.92	1.49	1.34	0.22	0.91	0.43
9	32	1.06	1.10	1.35	0.27	0.65	0.56
10	32	1.20	2.07	2.00	0.57	0.98	0.59
11	33	2.29	3.42	4.13	0.67	0.95	0.86
12	33	1.20	2.09	1.55	0.24	0.74	0.65
13	36	1.61	1.57	1.57	0.50	0.44	0.29
14	39	1.61	1.76	2.10	0.45	0.76	0.54
15	41	1.05	1.50	1.00	0.40	0.57	0.41
16	43	1.29	1.80	1.55	0.31	0.80	0.69
17	47	1.09	1.63	2.60	0.35	0.60	0.74
18	48	1.37	1.43	1.55	0.56	0.84	0.50
19	49	2.31	2.13	2.08	1.02	1.15	0.83
20	50	1.25	1.35	0.59	0.47	0.88	0.33
21	52	1.17	1.65	0.82	0.55	0.68	0.47
22	54	0.97	1.14	0.96	0.50	0.65	0.44
23	56	3.02	2.18	1.54	0.82	0.48	0.42
24	58	1.63	1.43	2.14	0.31	0.57	0.88
25	59	1.60	—	1.80	0.37	—	0.33
26	60	2.70	1.52	1.37	0.83	0.41	0.23
27	63	0.92	0.95	1.10	0.12	0.24	0.24
28	68	0.73	0.71	0.75	0.13	0.07	0.08
29	69	2.65	1.80	2.12	0.32	0.26	0.21
30	69	1.53	0.61	2.15	0.21	0.31	0.24
31	69	0.82	1.28	1.04	0.26	0.64	0.25
32	75	0.80	1.95	1.51	0.10	0.12	0.10
33	81	1.01	1.97	1.21	0.12	0.45	0.45
34	89	1.06	1.18	1.06	0.18	0.19	0.13
Means†		1.48±	1.65±	1.75±	0.40±	0.56±	0.43±
Mean		0.105	0.097	0.136	0.039	0.045	0.038
% increase over sleep†		—	21 ±7.2	21 ±8.1	—	61 ±14.9	30 ±13.8

* Expressed as 11-desoxycorticosterone equivalent.

† Numbers underlined with a single line give a "t" value for difference from the sleep determinations @ the 2% to 5% level of confidence, those underlined with a double line give a "t" value @ the 1% level of confidence or below.

tions of mean and women respectively are 50.6% and 57.0%; the corresponding PRL coefficients are 31.8% and 41.2%.

In a previous paper (Pincus *et al.* 1948a) we reported that although the neutral reducing lipid (PRL) and 17-ketosteroid excretion of men both exhibited a diurnal rhythm the *changes* in diurnal excretion of the two did not correlate significantly, suggesting that the secretion of the precursors of each might be under different influences. Furthermore absolute levels of output of each correlated only in the morning urine collections. Accordingly we have examined the relationship be-

tween the excretion of neutral reducing lipids and formaldehydogenic lipids. The correlation coefficients of these determinations for each collection period as well as the correlation coefficients for the percentage changes in morning and day collections have been determined, and in each set of data positive correlation coefficients are obtained. They vary between +0.21 and +0.62, and only one (+0.21 for the percentage change of morning output in the men's data) falls above the 5% level of confidence. We take these positive correlations to signify that formaldehydogenic and neutral reducing lipids vary quantitatively in the same way and that the diurnal changes also are similar for both.

In view of the large range of ages exhibited in the subjects of this study, we have examined the data for any notable relationship between age and the excretion values obtained. Dividing the men into 23–45 and 46–80 year groups gives no significant difference of output of either PRL or FL; a similar division of the women into 22–45 and 46–89 year age groups reveals no consistent difference (cf. Pincus *et al.* 1946). Nor do the positive correlations between PRL and FL disappear when the data for these age groups are examined. The implication is, therefore, that ageing does not differentially affect PRL or FL excretion.

The benzene:water partition of neutral lipids

To study further the possibilty of dissociation of formaldehydogenic and neutral reducing lipid excretion various types of urine collections were partitioned between benzene and water as described above. The data on the fractions obtained are presented in Table 3. It is notable that the partitioning procedure results regularly in the

TABLE 3. THE NEUTRAL REDUCING LIPID (PRL) AND FORMALDEHYDOGENIC LIPID (FL) OUTPUTS IN BENZENE: WATER PARTITIONS

Experiment No.	Period of collection	Neutral reducing lipid mg. per 24-hrs. in		% of combined fraction in		FL mg. per 24-hrs. in		% of combined fraction in	
		1 Crude "C" fraction	2 Combined benzene: water fraction	3 Benzene	4 H_2O	5 Crude "C" fraction	6 Combined benzene: water fraction	7 Benzene	8 H_2O
1	20.17 hrs.	1.83	1.41	50	50	0.20	0.18	39	51
2	24.00 hrs.	0.53	0.51	58	42	0.13	0.08	40	60
3	Night	—	2.30	62	38	0.39	0.28	46	54
	Morning	—	1.52	67	33	0.59	0.48	52	48
	Day	1.09	1.02	60	40	0.22	0.17	47	53
4	Prestress	2.67	2.30	74	26				
	Stress (ACTH)*	2.74	2.17	63	37				
	Poststress	2.43	1.95	65	35				
5	Prestress	2.84	3.04	65	35				
	Stress (ACTH)*	4.20	3.73	72	28				
	Poststress	3.22	2.68	68	32				

* 50 mg. adrenocorticotrophic hormone (ACTH) administered at beginning of stress period.

loss of both neutral reducing lipid (compare cols. 1 and 2) and formaldehydogenic lipid (compare cols. 5 and 6). Accordingly, we have expressed the partitioning of each into the benzene and water fractions as percentages (cols. 3, 4, 7 and 8) of the sum (cols. 2 and 6) of the two fractions. In different urine samples 50% to 74% of the neutral reducing lipid activity tends to segregate into the benzene fraction (col. 3) whereas 39% to 52% of the formaldehydogenic lipids are found in this fraction (col. 7). The data of Table 3 do not, however, indicate any notable departure from the typical partioning in different types of urine collection. For example, the PRL excreted after ACTH administration (experiments 4 and 5) does not partition differently from the prestress PRL. Nor does the partition of the formaldehydogenic substance in the diurnal samples of experiment 3 vary significantly with time of collection.

TABLE 4. SOME VALUES FOR OUTPUT OF NON-ALCOHOLIC REDUCING MATERIAL (PRL) PRESENT IN URINARY LIPID EXTRACTS

Experiment No.	Period	Mg. per 24-hrs. of total reducing lipid	Mg. per 24-hrs. of non-alcoholic reducing lipid	Percent of non-alcoholic in total reducing lipid
1	Night	1.39	0.33	24%
2	Prestress	1.71	0.52	30%
	Stress (ACTH)*	—	0.58	—
3	Prestress	1.76	0.37	21%
	Stress (ACTH)*	2.03	0.36	18%
4	Prestress	1.80	0.25	14%
	Stress (ACTH)*	3.52	0.62	18%
5	Desoxycorticosterone control	total 500 μg.	56 μg.	11%
6	Desoxycorticosterone control	total 320 μg.	30 μg.	9%

* 50 mg. adrenocorticotrophic hormone (ACTH) administered at beginning of stress period.

Non-alcoholic reducing lipids

A portion of benzene-soluble reducing lipid may be due in part to substances which are not typical adrenal cortex steroids. This is suggested by the fact that the FL tends to partition in larger amount into the aqueous extract which should concentrate the more highly oxygenated corticosteroids (Talbot *et al.* 1945). The steroids possessing typical adrenocortical activity are all alcholic substances by virtue of the C_{21} OH group. Accordingly they should be extractable as alcohols by the succinic anhydride reaction we have employed. In Table 4 we

present the reducing lipid (PRL) activity of the neutral non-alcoholic fractions of certain urine specimens. It will be noted that with amounts of desoxycorticosterone roughly comparable to the equivalents used in the urine aliquots approximately 10% failed to be extracted by the alcohol reagent (experiments 5 and 6) whereas larger proportions were found in this fraction after extraction of the urine samples (experiments 1 to 4). On the basis of the desoxycorticosterone control data, roughly 4% to 20% of the urinary fractions are probably non-alcoholic. Reducing activity of non-alcoholic substance in the phosphomolybdic acid reaction might be attributed to α,β unsaturated steroid ketones (Heard and Sobel 1946) or to miscellaneous non-steroidal substances. In any event it presumably contributes only a minor portion of the total reducing lipid activity, and shows no notable relation to ACTH stimulated output (experiments 2 to 4).

Phosphomolybdic acid and cupric ion reduction with various urinary extracts

Since the α,β unsaturation of certain typical steroids may be responsible for part of the reducing lipid values obtained by the Heard-Sobel method, opportunity for measurement of the reducing activity of the α-ketol side chain is offered by a study of the copper-reducing ac-

TABLE 5. PHOSPHOMOLYBDIC ACID REDUCING LIPID (PRL) AND CUPRIC ION REDUCING LIPID (CRL). URINARY OUTPUTS OF 16 WOMEN AND2 MEN

Subject No.	Age in years	Mg. per 24 hours					
		Phosphomolybdic acid reducing lipid*			Cupric ion reducing lipid*		
		Sleep	Morning	Day	Sleep	Morning	Day
1	32	1.51	0.97	4.13	0.45	0.66	1.19
2	55	1.27	1.33	1.90	0.45	0.80	0.46
3	56	2.72	2.10	1.30	1.18	0.57	0.30
4	61	0.79	1.13	1.20	0.31	0.40	0.36
5	62	1.43	1.62	1.85	0.61	0.38	0.35
6	67	1.41	2.52	3.00	0.32	0.37	1.05
7	70	1.34	1.30	1.60	0.90	0.59	1.05
8	72	1.39	2.49	1.80	0.51	0.86	0.58
9	76 (♂)	1.76	0.77	1.08	0.27	0.37	0.08
10	76	1.28	0.96	1.21	0.34	0.34	0.37
11	76	1.17	3.73	1.94	0.28	0.43	0.30
12	78 (♂)	0.82	—	1.74	0.27	—	0.33
13	79	1.25	3.04	2.84	0.42	1.11	0.60
14	82	0.74	0.68	0.72	0.30	0.36	9.24
15	82	1.62	2.20	2.80	0.45	0.74	0.53
16	83	2.84	2.10	1.21	0.71	0.46	0.59
17	90	1.13	1.20	1.38	0.80	0.45	0.27
18	91	1.51	0.95	0.98	0.19	0.14	0.21
Means†		1.44 ±	1.71 ±	1.82 ±	0.49 ±	0.53	0.49
Mean % increase† over sleep values		0.132	0.376	0.251	0.062	±0.058	±0.075
		—	18 ± 17.7	38 ± 14.3	—	21 ± 13.7	14 ± 17.7

* Expressed as 11-desoxycorticosterone equivalent.
† Underlining as in Tables 1 and 2.

tivity of the neutral urinary lipids. Heard and Sobel (1946) have demonstrated that cupric ion reduction by the Talbot *et al.* method is not effected by α,β unsaturated ketones lacking the α-ketol side chain at C_{17}.

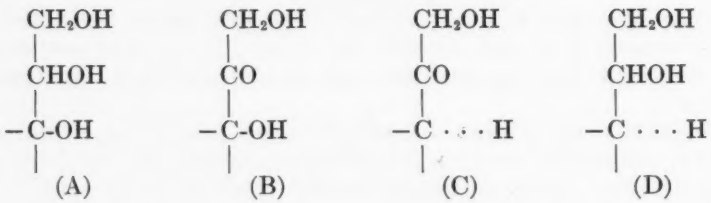
In Table 5 we present the data obtained on aliquots of diurnal rhythm urines measured by the phosphomolybdic acid and copper reagents respectively. Inspection of this table reveals consistently lower desoxycorticosterone equivalents by the latter method. In twelve of the eighteen subjects one or both of the non-sleep values exceeded the sleep values; this is true for fourteen of the eighteen in the CRL measurements. Increases are in the mean outputs for both PRL and CRL exhibited consistently for the morning and day values, but a significant percentage rise is had with these data only in the day values of the PRL measurements. It should be noted that the data of this table are contributed to chiefly by persons in the latter decades of life when there is ordinarily a less marked diurnal rhythm (Pincus *et al.* 1948b). The coefficients of variation are lowest for the PRL data in most of these measures, indicating a somewhat lower variability in the PRL method. Correlation coefficients between the CRL and PRL data of Table 5 have been calculated and are all positive (+0.25 to +0.78 in various measures), and all save one significant at the 1% level of confidence or below. This suggests that the same types of substances are measured by each method and that the output change of one tends to follow the output change of the other.

We have also measured the reducing lipid by these two methods before and after ACTH administration to three normal, healthy men. Both show corresponding increases: the mean PRL values were 1.62 and 2.78 mgm. desoxycorticosterone equivalents per 24 hours before and after ACTH respectively; the corresponding CRL values were 0.66 and 0.97. For the two methods these represent 72% and 52% output increases ascribable to the ACTH administration.

DISCUSSION

We believe that these data are notable for the demonstration of the entirely comparable output changes of the presumed urinary corticoid substance measured by the three methods employed. The values obtained by each method exhibit similar diurnal fluctuations and similar changes after ACTH administration. Furthermore, fairly consistent correlation of absolute output values is accompanied by remarkably consistent correlations between relative output changes. This is in contrast to the lack of correlation between 17-ketosteroid and neutral reducing lipid excretion (Pincus *et al.* 1948a).

Formaldehyde is generated from 20–21α-ketols or glycols. Among the adrenal cortex steroids this would include substances having the following side chains at C_{17}:



Reduction of cupric ion is accomplished by side chains of types B and C and reduction of phosphomolybdic acid by type B and C side chains and also by α,β unsaturated ketones. In spite of the fact that formaldehyde should be generated from two types of side chain (A and D) not active as the reducing substances as well as from the B and C types we regularly obtain much lower desoxycorticosterone equivalents on the basis of formaldehyde generation (cf. Tables 1, 2 and 3). A part of the excess measured by phosphomolybdic acid reduction may be accounted for by the non-alcoholic material in urine that acts on this reagent. On the basis of the data of 4 at most 20% of the neutral lipid of urine is non-alcoholic. We have recently had some evidence (Romanoff and Pincus 1949) that the neutral lipid fractions of human urine generate formaldehyde at a rate quite different from desoxycorticosterone. This may account for the lower equivalents obtained. Daughaday and collaborators (1948) give a brief report of formaldehydogenic steroid excreted by normal persons and find a range of 1.0 to 1.6 mgm. per day. This is much higher than the range we find (0.1 to 1.1 mgm. per day—see Tables 1 and 2), but they practice periodic acid oxidation on the total lipids or urine whereas we have removed the alkali soluble lipids.

Again, the consistently lower desoxycorticosterone equivalents obtained by the copper reducing method (see Table 5) require some explanation. While the non-alcoholic reducing lipid may account for a part of the discrepancy there still remains other material measured by the phosphomolybdic acid reagent which is apparently not measured by the copper reagent. Such substances may in fact be present in the extraction mixture, but we are inclined to ascribe the difference to the fact, noted by Heard and Sobel (1946), that many steroids are extremely insoluble in the usual aqueous alkaline copper solution.

In view of the close correlation of the determinations made by the three methods we have employed, we believe that each tends to measure the same type of urinary corticoid, at least with the types of urines that we have analyzed. Any consideration, therefore, of the method of choice may properly be concerned with such questions as ease of handling, degree of variability and so on. The phosphomolybdic acid reduction method seems to us to be the most convenient since it avoids certain manipulations requisite in the other methods (e.g. periodic acid oxidation), it is more sensitive and can therefore be em-

ployed with smaller urine aliquots. It presumably will include a certain amount of non-specific material not measured by the other methods, but does not apparently exclude certain poorly soluble steroids that are excluded by the copper reduction method. As far as the variability of the measurements are concerned those made by the phosphomolybdic acid reduction method seem, if anything, to be less variable than those obtained by the other two methods. Thus the coefficients of variation of the data of Tables 2 and 5 are generally lower for the phosphomolybdic acid reducing data. A benzene:water partition of the neutral lipid appears to offer no special advantage since the ratio between the titers of the two incident fractions shows no special alteration with either diurnal stress or ACTH administration.

It should be deduced from the foregoing that there is no special utility in fractionating the neutral lipids or in employing a particular type of chemical assay to such fractions. The larger proportion of FL in the aqueous phase of the benzene:water partition, for example, indicates a means of segregating such substance if its special identification is desired. Further partitioning (e.g., extraction of the ketonic substances with Girard's reagent) would lead to presumably more specific information about special types of compound. For routine assays seeking data on the particular type of adrenocortical activity index given by the corticoids of urine the method of Heard and Sobel offers the advantages of economy, rapidity and relatively low variability.

SUMMARY

Aliquots of diurnal urine collections from 34 men and 34 women were analyzed for phosphomolybdic acid reducing substance (PRL) and for substance generating formaldehyde on periodic acid oxidation (FL) in the neutral lipid fractions. Both types of substances exhibited similar diurnal variations in output and significant correlations were obtained between both the absolute levels of output of each and the percentage increases of each occurring in the morning and rest of day collection.

The division of the data into two age groups reveals no significant difference with age in basal outputs of PRL or FL. Nor do the data demonstrate any consistent difference in the absolute level or rate of change of output of PRL and FL with age. The positive correlation of PRL and FL output holds for both age groups.

The partition of the neutral urinary lipids between benzene and water was performed with five diurnal specimens and with urine specimens collected by three normal, healthy men before and after the administration of adrenocorticotrophic hormone. On the average somewhat more phosphomolybdic acid reducing substance segregated into the benzene. The ratio of benzene:soluble to water:soluble activity did not appear to alter significantly in the various diurnal periods or

after ACTH administration. In the fractionated diurnal specimens the formaldehydogenic neutral lipids were determined and on the average the aqueous extracts contained more FL than the benzene extracts.

Non-alcoholic PRL was extractable from various types of urine specimen. It varied between 4% and 20% of the total PRL, and did not change notably in proportion after ACTH administration.

A comparison of PRL and cupric ion reducing lipid (CRL) in diurnal urines of 16 women and 2 men revealed similar excretion trends. Absolute output levels and percentage changes in output PRL and CRL correlated positively.

In terms of desoxycorticosterone equivalent outputs of FL are least, CRL intermediate, and PRL greatest. The bases for these differences are discussed.

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THE EFFECT OF ADMINISTERED ADRENAL CORTICAL HORMONES ON THE LIVER GLYCOGEN OF NORMAL AND SCORBUTIC GUINEA PIGS.[1]

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CONSIDERABLE interest has developed concerning the role of cholesterol and ascorbic acid in the *in vivo* production of adrenal cortical hormones. This subject has been reviewed recently by Long (1947). Sayers, Sayers, Liang and Long (1946) have shown a rapid drop of the adrenal cholesterol and adrenal ascorbic acid in the rat and guinea pig with a simultaneous production of extra quantities of liver glycogen, when adrenocorticotropic hormone is administered. These decreases of cholesterol and ascorbic acid appear to take place only in the adrenal glands. These and other workers, including Levin (1945), have shown adrenal cholesterol changes in various states of stress. The indication, but not conclusive proof, is that cholesterol and ascorbic acid of the adrenal gland are utilized, either directly or indirectly, in the production of cortical hormones. Vogt (1948) in her studies of adrenal secretions in dogs and cats could show no correlation between the contents of ascorbic acid and of cortical hormone in the plasma obtained from the adrenal bloods. Lowenstein and Zwemer (1946) reported the, as yet unconfirmed, isolation of an active cortical hormone containing the ascorbic acid moiety. These workers stated that their ascorbic acid containing compound was about equal to 11-dehydrocorticosterone in carbohydrate metabolic activity. Related to these observations is the report of Giroud, Santa and Martinet (1940) that scorbutic guinea pigs showed a decrease in the cortical hormone content of the adrenal gland. Banerjee (1943) has shown lower liver glycogen values for scorbutic guinea pigs than for normal animals, which he believes may be due to the lowered amount of cortical hormones available for liver glycogen production.

A further indication that ascorbic acid is involved in some un-

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known way in the glycogenic activity of cortical hormones was obtained by McKee, Cobbey and Geiman (1947) from liver glycogen studies with scorbutic guinea pigs. In this preliminary report it was stated that the ascorbic acid deficient guinea pigs show a sluggishness in their rates of gluconeogenesis and of glycogenolysis. Furthermore the administration of cortical hormones to the vitamin deficient guinea pigs does not stimulate liver glycogen production as it does in normal pigs.

This work resulted from malarial studies by McKee and Geiman (1946) attempting to determine the reasons for the prevention of malarial parasite growth in ascorbic acid deficient monkeys. Although the results to date do not give a solution of the malarial question they do indicate an interesting interrelationship between ascorbic acid and adrenal cortical hormone activity.

EXPERIMENTAL

The guinea pigs used were male animals weighing initially 225 to 275 grams. They were obtained by the University animal farm from a local animal breeder. All the animals were maintained during the course of the experiment on a diet of Purina rabbit chow, shown by analysis to be deficient in ascorbic acid. The normal non-scorbutic pigs were given a daily aseptic subcutaneous injection of 10 mg. of ascorbic acid. The ascorbic acid solution, which contained 40 mg. of Mallinckrodt U. S. P. material per cc. of double distilled water, was stabilized to oxidation by sterile filtration through an ultrafine (U. F.) Corning sintered glass filter and storing in the cold room at 5°C. The animals were given food and water *ad libitum* and the growth rates determined by weighing the pigs daily. The guinea pigs on the stock diet plus ascorbic acid showed a normal growth rate over the 21 to 30 day period, while the animals receiving no vitamin C supplement began losing weight in about 14 to 21 days. This loss of weight, which was indicative of impending ascorbic acid deficiency, was confirmed, by analysis of plasma, liver, kidneys, and adrenals for ascorbic acid according to the methods of Mindlin and Butler (1938) and Bessey (1948).

The scorbutic animals were usually employed for the experimental manipulations, of manual feeding followed by fasting and hormone therapy, when they had lost about half of their weight gain during the experimental period. The normal guinea pigs, which were started on the diet at the same time as the scorbutic animals, were utilized simultaneously for the experimental work. For the experimental procedure all of the guinea pigs, both normal and scorbutic, were fed manually two times, four hours apart, and zero time was taken as the time of the second feeding. The last ascorbic acid injection was given the normal animals at the time of the first manual feeding. The food given was a balanced synthetic ascorbic acid deficient diet (McKee and Geiman,

1946). A liquid mix was prepared by adding a measured amount of tap water to an equal weight of food, stirring and agitating to keep suspended. The animals were held gently and allowed to drink (not forcefed) 5 cc. of the mixture. The process was repeated 4 hours later with another 5 cc. of the food mixture (total of about 25 calories). All the animals took the food very well.

The cortical hormones, Parke-Davis Eschatin (50 dog units per cc.) and 11-dehydrocorticosterone in oil (5 mg. per cc.),[3] were administered subcutaneously in divided doses. The Eschatin was given in 1.0, 0.5, 0.5, 0.5, and 0.5 cc. hourly doses and the animals sacrificed one hour after thelast dose. The 11-dehydrocorticosterone was given in two 0.5 cc. doses 2 hours apart and the animals sacrificed 2 hours after the second dose. The hormone administrations were started so that the animals would be sacrificed at the end of the 24 hour period of fast.

The animals were sacrificed by a sharp blow on the back of the head and the peritoneal and pericardial cavities quickly opened. Blood was withdrawn from the heart by means of a 5 cc. syringe and #20 hypodermic needle and placed in a centrifuge tube containing heparin (0.5 mg.). The tissues (liver, kidneys and adrenals) were rapidly removed and a representative sample from each lobe of the liver (total of about one gm.) quickly weighed into an Erlenmeyer flask containing 5 cc. of 35 per cent potassium hydroxide. A rubber stopper carrying an air condenser was fitted into the mouth of the flask and the flask and contents placed in a boiling water bath. After about 10 minutes, when the tissue was completely dissolved, the flask and contents were cooled. One cc. aliquots of the liver-potassium hydroxide were used for the determination of liver glycogen according to the method of Good, Kramer, and Somogyi (1933). The glucose obtained by 2N sulfuric acid hydrolysis of the twice precipitated glycogen was determined by the method of Folin and Wu, according to Folin (1929).

Simultaneous with the preparation of the liver sample for glycogen analysis, the adrenals and samples of the kidneys, liver and spleen were weighed, ground in glass mortars with 3 per cent metaphosphoric acid and analyzed for ascorbic acid according to the method of Bessey (1938). The blood was centrifuged and the plasma analyzed for ascorbic acid according to the method of Mindlin and Butler (1938). Although the plasma of the scorbutic pigs consistently contained no ascorbic acid, the plasma from normal animals was extremely variable in ascorbic acid content (0.0 to 0.8 mg. per cent).

RESULTS

In order to determine the earliest time at which the liver glycogen of the animals could be reduced to a minimum by fasting, a series of of normal and scorbutic animals were fasted 2, 12, 24, 36, 48 and 72

[3] The 11-dehydrocorticosterone in oil was kindly furnished by Merck & Company through the courtesy of Dr. Augustus Gibson.

hours. The values for liver glycogen, for adrenal, liver, kidney and spleen ascorbic acid, and the ratios of adrenal weight to body weight are given in Table 1. An interesting point regarding these data is the almost complete loss of ascorbic acid from the adrenals of the scorbutic pigs contrasted to the smaller losses in the vitamin C content of the other organs of the vitamin C deficient animal, particularly the liver which still contains 20 to 25 per cent of the normal (non-fasted) amount. Even in the 72 hour fasting period, the loss of ascorbic acid from the adrenals is faster than from the other organs (adrenals

TABLE 1. CHANGES IN ADRENAL-BODY WEIGHT RATIOS, ORGAN ASCORBIC ACID AND LIVER GLYCOGEN FOR NORMAL AND SCORBUTIC GUINEA PIGS AFTER VARYING PERIODS OF FASTING

Fasting period	Adrenal wt.	Ascorbic acid				Liver glycogen
	Body wt.	Adrenals	Liver	Kidney	Spleen	
hrs.	mg./gm.	mg. %	mg. %	mg. %	mg. %	gm. %
		Normal Guinea Pigs				
2	0.34 (5)*	126.5 (5)	30.2 (5)	12.2 (5)	34.9 (5)	5.990 (5)
12	0.37 (8)	118.0 (8)	22.6 (8)	10.6 (8)	41.2 (8)	2.650 (7)
24	0.42 (17)	115.8 (17)	20.1 (17)	8.9 (17)	34.1 (17)	0.041 (17)
36	0.43 (8)	102.8 (8)	18.0 (8)	8.0 (8)	29.9 (8)	0.039 (8)
48	0.50 (9)	93.8 (9)	19.4 (9)	8.4 (9)	33.5 (9)	0.131 (9)
72	0.48 (7)	70.2 (7)	19.7 (7)	7.5 (7)	25.9 (7)	0.061 (7)
		Scorbutic Guinea Pigs				
2	1.09 (5)	1.5 (5)	8.0 (5)	2.4 (5)	2.5 (5)	2.460 (5)
12	0.76 (6)	1.3 (6)	6.1 (6)	2.9 (6)	3.4 (6)	1.660 (6)
24	0.83 (16)	3.3 (17)	6.9 (17)	2.9 (14)	3.5 (17)	0.210 (17)
36	0.69 (5)	1.5 (8)	7.0 (8)	3.2 (8)	4.6 (8)	0.054 (8)
48	0.83 (5)	2.1 (6)	5.0 (6)	2.7 (6)	3.3 (6)	0.037 (6)
72	1.09 (10)	1.8 (10)	6.5 (4)	3.6 (4)	4.1 (4)	0.063 (8)

* The numbers in parentheses are the numbers of animals studied.

45, liver 35, kidneys 38 and spleen 26 per cent). Most important, however, is the absence in the scorbutic pigs of the normal rapid drop of liver glycogen to a minimal value in 24 hours and the subsequent (48 hour) rise. This normal compensatory increase in liver glycogen after 48 hours is due to the stimulation of the adrenals by the pituitary to produce gluconeogenic cortical hormones (Long, Katzin and Fry, 1940). Thus there appears to be in scorbutic guinea pigs a much slower than normal rate of glycogenolysis and gluconeogenesis, which is pictured graphically in Fig. 1.

In view of the work of Long, Katzin and Fry (1940), and of others showing the ability of adrenal hormones, particularly the 11 and 17 oxycorticoids, to produce liver glycogen in fasted animals, it seemed desirable to determine if the scorbutic guinea pigs had the ability to produce liver glycogen. As shown in Table 2 and Fig. 2 the Parke-Davis adrenal cortex extract, Eschatin, stimulated liver glycogen deposition. There was present 28 times more glycogen in the livers of the hormone treated animals than in the non-treated controls. Merck's 11-dehydrocorticosterone stimulated glycogen deposition with a pro-

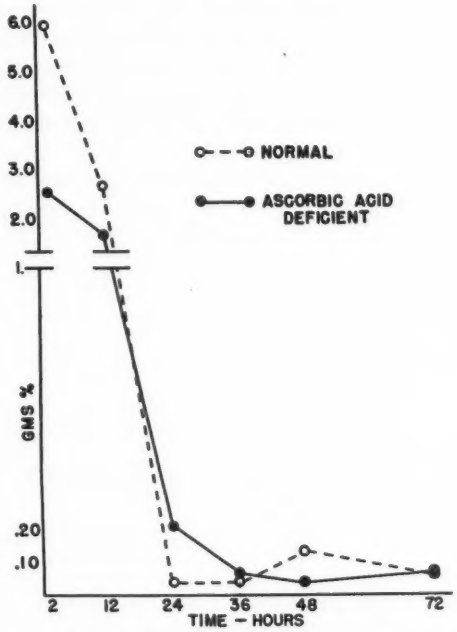

FIG. 1. Changes in liver glycogen during the fasting of normal and scorbutic guinea pigs.

duction of 8 times more glycogen than was present in the controls. Possibly the optimal effects were not obtained, particularly with the 11-dehydrocorticosterone in oil, due to inadequate time elapse following administration. The quantity of hormone available did not permit determining optimal conditions for administering the hormones.

TABLE 2. LIVER GLYCOGEN AND ORGAN ASCORBIC ACID AFTER INJECTION OF ADRENOCORTICAL HORMONES

Treatment	Adrenal wt.	Ascorbic acid				Liver Glycogen gm. %
	Body wt. mg./gm.	Adrenals mg. %	Liver mg. %	Kidney mg. %	Spleen mg. %	
		Normal Guinea Pigs				
No treatment	0.42 (17)*	115.8 (17)	20.1 (17)	8.9 (17)	34.1 (17)	0.041 (17)
Eschatin (Parke-Davis)	0.42 (11)	120.9 (11)	24.5 (11)	11.7 (11)	48.3 (11)	1.160 (11)
11-dehydrocorticosterone (Merck)	0.38 (4)	95.7 (3)	18.4 (3)	10.3 (3)	38.7 (3)	0.329 (3)
		Ascorbic Acid Deficient Guinea Pigs (19–23 days)				
No treatment	0.83 (16)	3.3 (17)	6.9 (17)	2.9 (14)	3.5 (17)	0.210 (17)
Eschatin (Parke-Davis)	0.94 (8)	1.5 (8)	4.6 (8)	1.8 (8)	2.5 (8)	0.065 (8)
11-dehydrocorticosterone (Merck)	0.86 (5)	2.7 (5)	6.1 (5)	1.1 (3)	3.2 (5)	0.117 (5)

* All animals were fasted 24 hours. The numbers in parentheses are the numbers of animals studied.

Contrasted to the striking deposition of liver glycogen in normal fasted animals, was the complete lack of deposition in scorbutic guinea pigs similarly treated with Eschatin and 11-dehydrocorticosterone (Table 2 and Fig. 2). In fact, there appeared to be even a slight, but possibly significant, hastening of the rate of glycogen removal from the livers of the scorbutic pigs.

Only one experiment with one group of animals has been carried out in an attempt to reverse the situation. In this experiment ascorbic acid (15 mg.) was administered during the period that the animals

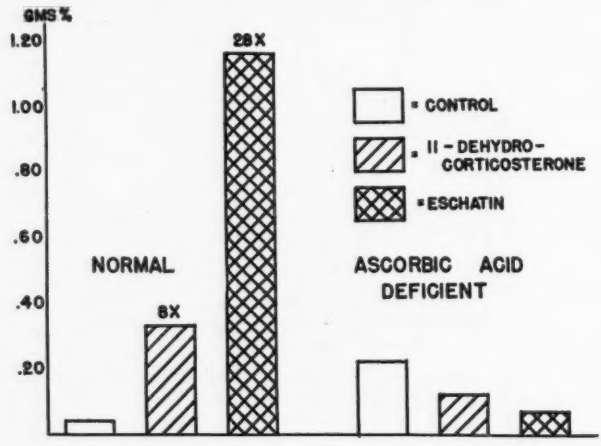

FIG. 2. The effects of Parke-Davis Eschatin and 11-dehydrocorticosterone on the liver glycogen of 24 hour fasted normal and scorbutic guinea pigs.

received the cortical hormones. No enhancement of liver glycogen deposition was obtained. Experiments are planned to determine when ascorbic acid must be administered to allow glycogen deposition with cortical hormone administration.

DISCUSSION

Under the conditions of our experiments the removal of ascorbic acid from the guinea pigs produced a condition incompatible with liver glycogen deposition when adrenal cortical hormones were administered. The reasons for this lack of cortical hormone activity are not known. Knowledge regarding the ease of reversibility with administered ascorbic acid of the above described condition in our scorbutic guinea pigs would be of value. Also the ability or inability of these animals to respond to injected adrenocorticotropic hormone would be helpful in evaluating the situation. The complement of available cholesterol, the suspected building block for the cortical hormones

also must be kept in mind. Analyses for adrenal and blood cholesterol were not made in these experiments.

As stated by Sayers, Sayers, Liang and Long (1946), "The lack of resistance of the scorbutic guinea pigs to various stresses, together with certain similarities between scurvy and adrenal cortical insufficiency, is at least worthy of further study. . . ."

The work of Banerjee (1943, 1944a, 1944b) and of Banerjee and Ghosh (1946) is of interest in regard to the influence of ascorbic acid deficiency on carbohydrate metabolism. They have shown a degeneration of the β-cells of the pancreas with a marked diminution of the insulin content of the gland, a mildly diabetic picture, and significantly lowered liver glycogen. The work of Colowick, Cori and Slein (1947) indicates an interrelationship of insulin and certain adrenal cortical hormones, as shown by an inhibition with adrenal cortical extracts of the hexokinase system from the muscle of rats made diabetic by alloxan. This inhibition could be counteracted by the addition of insulin. Whether or not the administration of insulin would stimulate glycogen deposition in scorbutic guinea pigs has to be determined.

SUMMARY

The fasting of normal guinea pigs produces a rapid drop of liver glycogen which within 24 to 36 hours approaches base line levels. There is a subsequent rise in the liver glycogen within 48 hours due to the action of the pituitary on the adrenal glands with the consequent production of cortical hormones and liver glycogen deposition. The cortical hormones (Eschatin and 11-dehydrocorticosterone) stimulate glycogen production in the fasted animal. This situation is strikingly different in the fasted ascorbic acid deficient guinea pig. (1) The liver glycogen drops more slowly. (2) There is not, within 72 hours, a secondary rise in the liver glycogen which is observed in normal animals due to pituitary stimulation of the adrenals. (3) The cortical hormones tested have no stimulatory effect on the production of liver glycogen. It was also observed that the ascorbic acid content of the adrenals drops faster than the vitamin content of spleen, kidneys, and liver during both periods of fasting and during the time that the animals are on an ascorbic acid deficient diet.

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INDUCTION OF PSYCHIC ESTRUS IN THE HAMSTER WITH PROGESTERONE ADMINISTERED VIA THE LATERAL BRAIN VENTRICLE[1]

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FOLLOWING estrone priming, the ovariectomized golden hamster responds to injections of progesterone in adequate doses by exhibiting psychic estrus as do also certain other common laboratory rodents, a fact first established by Frank and Fraps (1945) and subsequently verified by Kent and Liberman (1947) while studying vaginal smears associated with induced mating. While the threshold dose of progesterone necessary to effect psychic estrus when administered via a subcutaneous route has not been established, Frank and Fraps observed no mating among animals subjected to subcutaneous injection of 0.02 mg. of progesterone 24 to 48 hours after estrogen priming, but found that 0.05 mg. of progesterone was as effective in inducing mating as was 10 times this quantity. The purpose of the present paper is to report that doses of progesterone too small to effect psychic estrus when administered subcutaneously to ovariectomized, estrogen-primed females may be entirely adequate in inducing typical mating responses when introduced directly into the ventricles of the brain. In the present experiments no attempt was made to determine the minimum dose of progesterone necessary to effect psychic estrus by either the subcutaneous or the ventricular route. The possible significance of the findings will be discussed briefly.

MATERIALS AND METHODS

Animals utilized in the present experiment had been ovariectomized two weeks (in a few cases, slightly longer) before receiving the priming regimen of estrone. At the time of the initial estrone injection the vaginal smear was typical of castrate animals and none had exhibited estrous cycles or mating responses in the presence of males subsequent to ovariectomy. The regimen of estrone consisted of a series of injections of 50 μg estrone daily for 6 days, a regimen sufficient to produce, on or before the sixth day, typical vaginal estrous smears devoid of leucocytes and composed of epithelial cells with polyhedral squamous cells predominating.

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Twenty-four hours after the last dose of estrone the animals were tested for mating responses. Of 63 animals employed to this point, one exhibited a mating response as a result of the effects of the estrone, and this animal was discarded. Both Frank and Fraps, and Kent and Liberman have shown that the effect of estrone in the induction of psychic estrus is erratic, and the exhibition of psychic estrus by this animal is not unexpected at the dosage level herein utilized. Two animals were subsequently lost in trephining.

Sixty animals were utilized in three groups as follows: Fifteen animals received subcutaneously 0.025 mg. progesterone (crystalline synthetic) in 0.05 cc. of olive oil previously neutralized with sodium bicarbonate and extracted with ether; 30 animals received 0.025 mg. progesterone in 0.05 cc. oil directly into the right lateral ventricle; 15 animals received 0.05 cc. oil alone, directly into the right lateral ventricle. Several additional animals were injected via the ventricle with oil containing India ink to determine the distribution of the granules and therefore the distribution of progesterone within the ventricular system. These latter animals were autopsied immediately after injection. The ink granules were found in the contralateral ventricle, in the hypothalamic region of the 3rd ventricle, and in the iter. It is presumed that, in the test animals, the progesterone underwent a similar dissemination.

Injection into the ventricle was via a trephine hole placed 6 hours earlier in the right parietal bone half way between the coronal and lambdoidal sutures and 1.5 mm. lateral to the sagittal suture. The animals were ambulatory and alert less than 10 minutes after trephining. A 26 gauge intradermal needle attached to a 1 cc. tuberculin syringe was directed ventrad and slightly laterad through the hole, the meninges, and the cerebral cortex while the animal was under light ether anesthesia.

The brains of the test animals receiving progesterone via the ventricles were fixed in ammoniacal alcohol after observations had been completed at the end of twenty-four hours and were sectioned transversely by hand. The pathway of the needle through the cerebral cortex and into the ventricle was observed in the sections with the aid of low power lenses. In most cases the needle penetrated the right lateral ventricle and the postero-dorsal-most extent of the right inferior horn. Trauma was probably not avoided entirely in the adjacent ventricular walls. In no instance had the needle been directed mediad, and as far as could be ascertained by gross examination the thalamus suffered no direct damage.

OBSERVATIONS

Of the 15 animals which received subcutaneously 0.025 mg. progesterone in oil all gave negative responses when subsequently tested for mating reactions. Testing commenced 20 minutes after the injection and a response was considered negative when the female failed to exhibit lordosis after 4 hourly exposures to a male of approximately 10 minutes each, followed by a single exposure 10 hours later. Antagonism on the part of the female necessitated the removal of the male before 10 minutes had elapsed in all except one instance. In previous experiments utilizing larger doses (0.1 mg.) of progesterone the present authors (1947) were able (after 3 hours) to elicit 33 positive responses in 33 trials. Frank and Fraps described the latent period of response to progesterone as not more than 2 or 3 hours.

Of the 15 animals receiving 0.05 cc. oil alone into the ventricle, all exhibited negative mating responses.

Of the 30 animals receiving 0.025 mg. progesterone in oil into the ventricle, all exhibited positive mating responses (lordosis and mating) at the end of 1 hour. Sixteen animals in this group were tested for mating responses 1 hour after injection and were positive at that time. One animal was tested at 50 minutes, 4 at 30 minutes, 2 at 25 minutes, 1 at 20 minutes, and 6 at 10 minutes, and all except one of the six in the last group were positive when tested. The animal which refused to exhibit lordosis 10 minutes after injection was positive when re-tested 5 minutes later. Lordosis was exhibited from 15 seconds (1 animal) to 2 minutes (7 animals) after introduction of a male into the cage with the test female for the first time after injection, most of them responding in 30 to 90 seconds. When tested at subsequent hourly intervals the reaction time to the male invariably decreased until, on the third hour, the female typically exhibited lordosis 10 seconds after exposure. The reflex following intraventricular injection was, in our experience, particularly strong, although no objective measurements were made. In one instance in which the male was withdrawn from the cage before having mounted a lordotic female the latter remained in rigid lordosis for 40 minutes.

Observation of vaginal smears 24 hours after the animals received 0.025 mg. progesterone via the ventricles revealed that these animals did not exhibit a vaginal response equivalent to that elicited in previous experiments (Kent and Liberman, 1947) in which the animals received, subcutaneously, a larger dose (0.1 mg.) of progesterone. The latter animals within 24 hours exhibited a condition reminiscent of typical diestrous smears of unoperated females, characterized by the presence of large numbers of leucocytes. In the present experiments utilizing smaller doses of progesterone leucocytes appeared in small numbers only and the smears apparently were tending toward an ultimate castrate condition. Jones and Astwood (1942) have shown in the rat that, if the dosage of progesterone is adequate, diestrus will follow an estrous smear even in the presence of excess estrogen.

Numerous investigations chiefly on the guinea pig have indicated a likelihood that one or more hypothalamic nuclei may be indispensable elements in the complex neural and hormonal integrating mechanism which ultimately brings about mating behavior at a specific stage of the estrous cycle in rodents (Bard, 1940; Brookhart *et al.*, 1940, 1941; Dey *et al.*, 1940, 1942; Dey, 1943). In the present experiment the following facts seem significant: 1. A dose of progesterone too small to effect psychic estrus when administered subcutaneously into the body wall will, if injected directly into the neurocoele of the forebrain of animals pretreated with estrone, facilitate the exhibition of a strong mating reflex. 2. The latent period of response to the progesterone is relatively short (10 minutes or longer). On the basis of these observations it is suggested that, in the intact animal, progesterone may act

(in a manner not understood) directly upon one or more nervous centers causing females to enter a physiological state known as psychic estrus. This state is manifest by the exhibition of postural reflexes initiated by adequate environmental stimuli chief of which, in a natural environment, are the presence and behavior of the male. Such an interpretation precludes a role of any other endocrine organ or secretion (with the exception of estrone the role of which has not been elucidated) in the exhibition of mating activity on the part of the female hamster once progesterone, elaborated by a preovulatory follicle (perhaps under the stimulus of prolactin in the rat as suggested by Everett in 1944) affects the proper nervous centers. Further integrating mechanisms resulting in lordosis are, therefore, envisaged as entirely nervous in nature.

SUMMARY

Fifteen ovariectomized golden hamsters pretreated with estrone received subcutaneously 0.025 mg. progesterone in oil and, as expected on the basis of previous observations of the size of dose necessary to effect psychic estrus by subcutaneous administration, exhibited no subsequent mating reaction when presented with males. Thirty additional animals received 0.025 mg. progesterone in oil directly into the right lateral ventricle, and all exhibited positive mating responses within one hour or less. Fifteen animals received an equivalent amount of oil alone directly into the ventricle and these exhibited no mating responses. The progesterone is believed to have been distributed in the contralateral ventricle, in the hypothalamic region of the 3rd ventricle, and in the iter. On the basis of these experiments it is suggested that progesterone may act directly upon one or more cranial nuclei facilitating, in an unknown manner, the manifestation of reflex mating activity in the female hamster, thus effecting the physiological state known as psychic estrus. In the present experiment vaginal smears typical of diestrus did not occur subsequent to progesterone administration although in earlier experiments utilizing larger doses of progesterone diestrual smears were observed.

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THE EFFECT OF THYROXINE ON THE FEMALE REPRODUCTIVE SYSTEM IN PARABIOTIC RATS[1]

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NUMEROUS reports in the literature have demonstrated that experimentally induced hyperthyroidism may exert definite inhibitory effects on the female reproductive system. A decrease in the number of ovarian follicles after large dosages of desiccated thyroid has been observed in rats by Da Costa and Carlson (1933) and in rabbits by Chu and You (1945). Irregularity in or total cessation of the estrous cycle in hyperthyroid rats has been noted by Cameron and Amies (1926), Reiss and Pereny (1928), Weichert and Boyd (1933), Van Horn (1933) and Halpern and Hendryson (1935). The effectiveness of various injected gonadotrophins in stimulating the ovaries of immature rats has been markedly reduced by thyroid feeding or thyroxine injection (Fluhman 1934; Tyndale and Levin, 1937; Tolksdorf and Jenson, 1939).

An increase in the gonadotrophic potency of the hyperthyroid rat pituitary gland as indicated by assays in immature animals of the same species has been demonstrated by Evans and Simpson (1930), Cohen (1935), and Membrives (1938). A similar condition has been observed in the rat hypophysis after castration (Evans and Simpson, 1929, and others). Cytological studies on the pituitary glands of thyroid-fed rats by Sevringhaus, Smelser and Clark (1934) have shown an increase in number and vacuolation of basophils and an increase in granulation of acidophils. The basophilic vacuolation is like that which occurs after castration. Sevringhaus (1939) has reviewed the literature concerned with the effects of hyperthyroidism on pituitary reproductive physiology and cytology.

The way in which hyperthyroidism exerts its effects on the reproductive system is not clearly understood. Most of the literature cited above indicates that the results obtained were due to specific actions of thyroid substances on the ovaries or the pituitary glands rather than to general metabolic disturbances. Smelser (1939) has suggested that the decreased testicular activity observed in hyperthyroid male rats was caused by an increased threshold of response of the testis to gonadotrophic hormones. On the other hand, Chu and You (1945) have

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postulated that the thyroid gland directly controls the gonadotrophic activity of the hypophysis in rabbits, hyperthyroidism inhibiting the production of follicle-stimulating hormone and inducing the elaboration of luteinizing hormone.

The technique of parabiosis has been used frequently in studying pituitary-gonadal interactions. Kallas (1930), Hertz and Meyer (1937), Bunster and Meyer (1938), and Biddulph, Meyer and Gumbreck (1940) have shown that when an ovariectomized female rat is placed in parabiosis with a normal female rat the ovaries of the latter undergo marked stimulation because of a gonadotrophic hypersecretion from the pituitary gland of the ovariectomized parabiont. The present parabiosis experiments were undertaken to study the effect of thyroxine on this pituitary-ovary interrelationship in an attempt to determine the nature of its action on the reproductive system.

METHODS AND PROCEDURE

A total of 157 pairs of litter-mate female albino rats were joined in parabiosis according to the method of Bunster and Meyer (1933) except that metal skin clips were used instead of silk sutures.[2] The operations were performed between the 31st and 33rd days of life upon animals weighing in excess of 70 grams. In all cases the right hand partners were ovariectomized at the time of parabiotic union. The ovaries of the left hand partners remained intact to serve as a measure of the pituitary gonadotrophic effectiveness of the ovariectomized parabionts. Twenty-three of the pairs were reserved as uninjected controls. The remaining 134 pairs were injected once daily for periods of 10 days with dosages of thyroxine[3] ranging from 0.025 mg. to 0.15 mg. In 68 of the experimental pairs the ovariectomized right hand partners were injected and in 66 pairs the intact left hand partners received the injections. Both experimental and control pairs were autopsied on the 11th day after the operations at which time the ovaries of the normal partners were freed of extraneous tissue and weighed to the nearest milligram. The qualitative response of the ovaries, the degree of uterine stimulation, and the presence or absence of vaginal canalization were also noted.

The pituitary glands of most of the thyroxine-treated and control pairs were pressed out individually between microscope slides and stored in a desiccator. After periods of storage not exceeding 6 months, the dried pituitary tissue was powdered and weighed out in 2.5 mg. quantities. Usually from 3 to 5 desiccated glands were required to make up this weight of powder. The glands of intact partners were always prepared separately from those of ovariectomized partners and glands of intact partners with unstimulated ovaries were never pooled with those of intact partners showing ovarian stimulation. Each weighed unit of powder was suspended in 5 cc. physiological saline and thoroughly shaken. The glands were assayed in 21 day old female mice, each mouse receiving 0.5 cc. of the suspension twice daily for periods of 4½ days. The pituitary powder from a given group of ovariectomized partners was assayed simultaneously in litter-mate mice with that

[2] The data were obtained during the years 1939–1941.

[3] Roche-Organon.

obtained from their intact parabiotic mates. In most cases a third litter mate mouse served as an uninjected control animal. All mice were autopsied on the morning of the 6th day after the beginning of the injections. The ovaries and the uteri were weighed to the nearest tenth of a milligram. Assays were also performed on pituitary glands from 41–43 day old ovariectomized and normal single female rats which had received no thyroxine.

Immediately before autopsy, several of the thyroxine-treated and control pairs were placed under ether anesthesia and bled from the heart, the blood from ovariectomized and normal partners being collected separately. The blood was centrifuged and the serum obtained was stored in a frozen condition. The serum was assayed in 21 day old female mice, each animal receiving 0.5 cc. 4 times daily for $4\frac{1}{2}$ days. Thus, each mouse received a total dosage of 9 cc. serum. As in the pituitary assays, serum from ovariectomized and normal donors was injected separately in litter-mate mice. Autopsies were performed on the morning of the 6th day after the beginning of the injections. Ovarian and uterine weights were recorded and the condition of the vaginas was noted.

RESULTS AND DISCUSSION

The toxic effect of thyroxine was quite marked, particularly in those animals receiving the highest daily dose. Thirty-three of the 134 experimental pairs died before the end of the injection period. Data from these animals are not included in the following discussion. Among the surviving thyroxine-treated pairs there was usually a definite body weight loss, although there were several exceptions and the majority of animals appeared to be in good health. There were no deaths among the control pairs and most of them gained a few grams in weight during the period of parabiosis.

The data in Table 1 show that in approximately 68% of the surviving experimental pairs, thyroxine prevented ovarian hypertrophy in the intact partners. In these animals the ovaries weighed less than 20 mg., being no heavier than those of normal single rats of the same age. Qualitatively, the ovaries contained only small follicles. The uteri in all cases were unstimulated and the vaginas of both partners were closed. In the control group, only 2 animals (9%) showed a negative ovarian response. The intact partners of the remaining untreated pairs exhibited definite ovarian and uterine stimulation. Statistically, the difference in ovarian weight averages (26 mg. for the thyroxine-treated pairs and 80 mg. for the control pairs) is highly significant.

An attempt was made to obtain a quantitative measure of the effectiveness of thyroxine in preventing ovarian hypertrophy by injecting it at 4 dose levels. It will be noted (Table 1) that the lowest daily dose of 0.025 mg. was least efficient in this respect. When the amount was raised to 0.05 mg. per day a much higher incidence of ovarian inhibition occurred. Doses of 0.10 mg. and 0.15 mg. were also effective in preventing ovarian hypertrophy but were not significantly more so than the 0.05 mg. dose. Amounts greater than 0.15 mg. were

TABLE. 1 THE INCIDENCE OF OVARIAN INHIBITION AND OVARIAN WEIGHT AVERAGES OBTAINED IN THE INTACT PARTNERS OF THYROXINE-TREATED AND CONTROL PARABIOTIC RATS

Partner injected	Daily thyroxine dose (mg.)	Numbers of pairs	Pairs with unstimulated ovaries	Average ovarian weights (mg.)
Intact	0.025	9	2	55 ± 11.2*
	0.05	9	8	32 ± 19.6
	0.10	23	16	21 ± 4.4
	0.15	8	6	21 ± 5.7
Totals intact partners injected		49	32	29 ± 8.7
Ovariectomized	0.05	10	2	43 ± 10.1
	0.05	19	16	18 ± 3.5
	0.10	15	11	27 ± 9.3
	0.15	8	8	12 ± 1.2
Totals ovariectomized partners injected		52	37	25 ± 6.5
Totals thyroxine-treated pairs		101	69	26 ± 7.3
Control pairs		23	2	80 ± 9.1

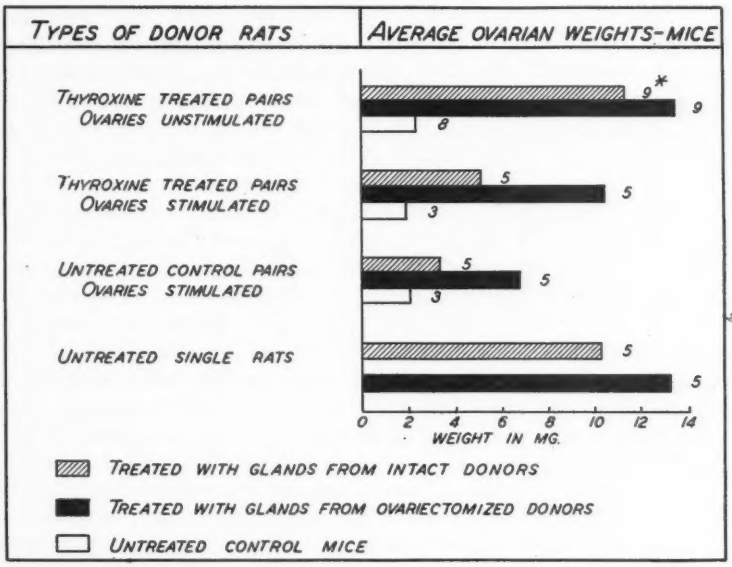
* Standard error.

not administered because of the toxic effect of thyroxine. Thus, total ovarian inhibition was not obtained with any of the 3 highest doses. Small numbers of pairs at each level were, for no obvious reason, comparatively resistant to thyroxine and showed varying degrees of ovarian and uterine stimulation. The higher resistance of these animals is also indicated by their relatively slight body weight loss. This averaged 7 gm. per pair in the animals with stimulated ovaries as compared to 15 gm. per pair in the rats showing no ovarian stimulation. Meyer and Hertz (1937) and Biddulph, Meyer and Gumbreck (1940) produced complete ovarian inhibition in intact parabionts by administering estrone, estradiol, and estriol to the ovariectomized partners. Thyroxine, therefore, was not as effective as estrogens in preventing ovarian hypertrophy.

Administration of thyroxine to the intact parabionts produced a negative ovarian response in 65% of the animals so treated. When the hormone was administered to the ovariectomized partners, 71% of their normal parabiotic mates showed unstimulated ovaries. The similarity of these results indicates that thyroxine passed freely across the parabiotic union, inducing a hyperthyroid condition in both partners. As a result, the inhibitory effect of thyroxine on the ovaries of the intact parabionts was equally manifest regardless of which partner was injected. In this respect, the action of thyroxine differs from that of the sex hormones. Biddulph, Meyer and Gumbreck (1941) and Shipley, Meyer and Biddulph (1943) have shown that many times the minimum stimulating doses of estrogens and androgens must be

administered to one parabiont before evidence of transfer appears in the other.

The parabiosis data demonstrate that thyroxine was effective in preventing ovarian hypertrophy in the intact parabionts, but they provide no direct evidence as to the mechanism of this action. Under the conditions of the experiment, thyroxine may have: (1) inhibited the pituitary glands by preventing the production or release of gonadotrophic hormone; or (2), rendered the ovaries of the intact parabionts refractory to gonadotrophic stimulation. In order to study



* Number of mice in assay.

FIG. 1. Average ovarian weights of immature mice injected with 2.5 mg. suspensions of dried pituitary powder from thyroxine-treated and control parabiotic rats and from untreated ovariectomized and intact single rats.

these possibilities, assays were performed on the pituitary glands of thyroxine-treated and control pairs.

Average ovarian weights of mice receiving 2.5 mg. amounts of dried pituitary tissue from various groups of intact and ovariectomized parabionts are represented graphically in Figure 1. Also shown are the results of assays of glands from ovariectomized and normal single rats.

Assay data from normal donors show that pituitary glands from the intact partners of thyroxine-treated pairs with unstimulated ovaries produced marked ovarian hypertrophy in mice. In this respect they were of about the same degree of potency as glands from untreated

single rats of the same age having immature ovaries. However, they were more than twice as effective in the mouse assays as pituitaries from the intact partners of thyroxine-treated pairs with stimulated ovaries and more than 3 times as potent as glands from the corresponding partners of untreated control pairs in which the ovaries had also undergone marked hypertrophy.

It is evident from these data that thyroxine treatment did not prevent the production of gonadotrophic hormone by the pituitary glands of the intact parabionts. Instead, the factor controlling pituitary gonadotrophic potency in these animals appeared to be the functional condition of their ovaries, or, in other words, the blood level of estrogens. Thus, in both experimental and control parabionts with enlarged ovaries the pituitary glands were low in gonadotrophic content. In other parabiosis experiments, Meyer, Biddulph, and Finerty (1946) have also provided evidence that the production of gonadotrophin by the pituitary glands of intact parabionts is inhibited by estrogens from their own stimulated ovaries. In most hyperthyroid pairs, on the other hand, unstimulated ovaries in the intact partners were associated with relatively potent pituitary glands. A similar condition existed in immature single rats. These data suggest that thyroxine decreases the sensitivity of the ovaries to gonadotrophin and as a result the blood estrogen level is low. This favors production and perhaps storage of gonadotrophin by the pituitary gland.

In the ovariectomized donors the same relative order of pituitary gonadotrophic potencies prevailed as in the intact donors. Thus, glands from the ovariectomized partners of thyroxine-treated pairs with unstimulated ovaries and hypophyses from ovariectomized single rats produced the greatest ovarian response in mice. They were only slightly more potent than glands from thyroxine-treated pairs with stimulated ovaries, but were twice as effective as pituitaries from ovariectomized control parabionts. Here again, there was no evidence of a decreased production of gonadotrophic hormone as a result of thyroxine treatment. In most hyperthyroid pairs, relatively potent pituitary glands in the ovariectomized partners were associated with unstimulated ovaries in the normal parabiotic mates. The reverse was true in control pairs and in those experimental pairs which were relatively resistant to thyroxine.

A possible interpretation of these data is based on the concept of utilization of gonadotrophin by the ovaries as suggested by the experiments of Seidlin (1940) and Heller, Heller, and Sevringhaus (1942). It can be postulated that the ovaries of the intact partners of control pairs were comparatively sensitive to gonadotrophic stimulation. Consequently, they became enlarged by removing and metabolizing gonadotrophin from the blood stream. This resulted in an increased output of hormone from the pituitaries of the ovariectomized partners and a lowering of their gonadotrophic content. Supporting this con-

cept are the triple parabiosis experiments of Plagge (1948) which indicate that the gonadotrophic secretion from the castrated left hand partner was so completely utilized by the enlarged ovaries of the middle partner that ovarian hypertrophy did not occur in the right hand member of the trio. In most hyperthyroid pairs it can be supposed that the ovaries of the intact partners were insensitive to stimulation. Hence there was little utilization of gonadotrophin from the hypophyses of the ovariectomized parabionts. This resulted in a storage of hormone in the glands of those animals and a higher potency at assay. In this respect, they resembled the glands of ovariectomized single rats.

The results of the pituitary assays pointed to a direct inhibitory effect of thyroxine on the ovaries. However, the possibility remained that thyroxine had prevented the release of gonadotrophin from the

TABLE 2. INDIVIDUAL AND AVERAGE OVARIAN AND UTERINE WEIGHTS OF MICE INJECTED WITH BLOOD SERUM FROM THYROXINE-TREATED AND CONTROL PARABIOTIC RATS

Types of Donor Rats	Recipient mice								
	Serum from Ovariectomized partners			Serum from Intact partners			Untreated control Mice		
	Ov. Wt. (Mg.)	Ut. Wt. (Mg.)	Vag.	Ov. Wt.	Ut. Wt.	Vag.	Ov. Wt.	Ut. Wt.	Vag.
Thyroxine-treated pairs:	2.2	16.0	Open	2.2	15.7	Closed	2.4	10.8	Closed
Ovaries of intact	3.1	18.0	Open	3.0	14.0	Open	2.8	15.0	Closed
partners unstimulated	3.1	22.3	Open	2.1	12.4	Closed	1.6	13.5	Closed
Averages hyperthyroid serum	2.8	18.8		2.4	14.0	—	2.3	13.1	—
Control pairs:	2.0	65.3	Open	1.2	12.7	Closed	—	—	—
	1.4	20.0	Open	1.8	13.7	Closed	1.9	13.6	Closed
Ovaries of intact	4.3	13.8	Closed	3.0	12.9	Closed	3.3	12.5	Closed
	4.8	15.4	Closed	3.6	11.7	Closed	3.3	12.5	Closed
partners stimulated	2.1	21.4	Open	1.8	15.9	Open	1.5	16.0	Closed
Averages control serum	2.9	27.2		2.3	13.4		2.2	13.7	

pituitary glands, thereby lowering its concentration in the blood stream. To test this assumption, serum assays were performed.

Table 2 shows that serum from the ovariectomized partners of both hyperthyroid and control pairs produced no definite ovarian hypertrophy in mice. However, some uterine stimulation occurred, and in 6 of the 8 animals receiving this treatment the vaginas were open at autopsy. Assays of serum from intact parabionts of both experimental and control pairs were negative with the exception of one mouse in each group in which the vagina was open. The slightly greater serum potency of ovariectomized partners may have been the result of an accumulation of gonadotrophin in the blood of those animals caused by a retarded passage across the parabiotic union.

The serum assays, although limited, show no significant difference in blood gonadotrophic content between experimental and control

pairs. The somewhat higher uterine weight average obtained in mice treated with ovariectomized control serum was due to one animal in which the uterus had undergone marked hypertrophy. These data are in accord with the more extensive assays of Smelser (1939) who found no difference in gonadotrophic effectiveness between hyperthyroid and control serum from castrated rats. It would appear, therefore, that thyroxine administration did not change the rate of pituitary gonadotrophic secretion.

In conclusion, it can be stated that the female reproductive system in parabiotic rats was affected by thyroxine in a manner similar to that reported in the literature for single rats. The most striking manifestations were ovarian inhibition in the intact parabionts accompanied by an increased pituitary gonadotrophic potency in both partners. This increased potency in the intact partners appeared to be caused by the low blood estrogen levels resulting from hypofunctional ovaries. Evidence is provided that the higher potency of glands from ovariectomized hyperthyroid parabionts was due to a lack of utilization of their secretions by the ovaries of the intact partners. The serum assays indicate that the unstimulated ovaries of experimental pairs were under the influence of about the same blood concentrations of gonadotrophin as were the stimulated ovaries of control pairs. This situation is comparable to that observed in the previously cited experiments of Fluhman (1934) and others, in which administration of thyroid substances to single rats reduced the effectiveness of injected gonadotrophins in stimulating the ovaries. Thyroxine, therefore, probably produces ovarian inhibition in the rat by directly decreasing the sensitivity of the ovary to gonadotrophic stimulation.

SUMMARY

Administration of thyroxine to ovariectomized female-intact female parabiotic rats prevented ovarian hypertrophy in 68% of the intact partners. This effect occurred regardless of which partner was injected.

Pituitary glands from both the intact and ovariectomized partners of thyroxine-treated pairs with unstimulated ovaries had greater gonadotrophic activity than glands from the corresponding partners of either hyperthyroid or control pairs with stimulated ovaries.

Hyperthyroid blood serum contained the same amount of gonadotrophic hormone as control serum. However, serum from the ovariectomized partners of both groups was slightly more potent than serum from the intact partners.

Thyroxine appears to exert its inhibitory effect on the female reproductive system by decreasing the sensitivity of the ovary to gonadotrophic hormone.

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HISTOLOGICAL, CYTOCHEMICAL AND PHYSIOLOGICAL OBSERVATIONS ON THE REGENERATION OF THE RAT'S ADRENAL GLAND FOLLOWING ENUCLEATION[1]

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WITH THE accumulation of evidence for a functional zonation of the adrenal cortex, as postulated by Swann (1940) and supported by Sarason (1943) and by Deane and Greep (1946), the origin and functional capacity of new cortical tissue arising after enucleation of the gland acquire new significance.

Adrenal enucleation involves slicing open the capsule and extruding the medulla and most of the cortex (Ingle and Higgins, 1938). If both glands are so enucleated, or if one gland is enucleated and the other totally removed, regeneration of cortical tissue takes place rapidly so that the original volume is restored in about a month. (Because of the regeneration of the cortex, the operation is generally termed demedullation.) The regeneration is dependent upon the presence of the pituitary.

The origin of the new parenchymal tissue is still a matter of controversy. Originally Ingle and Higgins described regeneration as taking place from the capsule, but Higgins (personal communication[2]) has informed us that they meant the capsular region, i.e., the capsule and the subcapsular cortical cells. Because of the reports of Zwemer, Wotton and Norkus (1938), Baker and Baillif (1939), Gruenwald and Konikov (1944) and Baxter (1946), however, it is often assumed that the origin of new cortical cells is principally from fibroblasts in the capsule. In addition to this problem of the involvement of the cap-

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[1] This work was done in part under a grant to the Department of Anatomy from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

[2] Statement from Dr. G. M. Higgins, August, 1948: "In our studies, a number of years ago, upon the regeneration of the adrenal gland Dr. Ingle and I undertook a study of cortical regeneration in adrenal glands of rats from which the medulla and most of the cortex had been expressed. It is obvious that such procedures can not entirely free the capsule of all of the adjacent cortical tissues, so that small islands of glomerulosal cells always remained adherent to the capsule. Whereas we used the term capsule to define that portion of the adrenal which remained in place upon enucleation, it is certain that the number of glomerulosal cells which persisted formed foci from which, at least very largely, subsequent regeneration of cortical tissue occurred."

sule, the question further arises as to whether or not peripheral tissue forms a cortex capable of secreting hormones which influence both salt and sugar metabolism. This is of especial importance because evidence is accumulating to show that the zona glomerulosa secretes those hormones which influence salt and water metabolism (Deane, Shaw and Greep, 1948), whereas the zona fasciculata secretes those which affect protein and carbohydrate metabolism (Bergner and Deane, 1948). The present study was undertaken to determine, in so far as possible, both the origin of the new tissue and its functional characteristics. Histological, cytochemical and physiological methods have been employed.

MATERIAL AND METHODS

The animals used in these experiments were adult (200 gm.) male rats of the Long-Evans strain. Bilateral adrenal enucleations were performed under ether anesthesia. Each adrenal gland was exposed and its capsule sliced open at one pole. With small curved forceps slight pressure was exerted at the opposite pole, causing the extrusion of a solid core, which was removed. The capsule was then "milked" between closed forceps to remove all parenchymal tissue possible. The capsule of the enucleated gland was restored to position in order to permit regeneration. Three types of experiments were performed.

1) *Histology and cytochemistry.* Groups of 3 rats were killed 1, 3, 8, 18 and 32 days after operation.

One adrenal from each group of 3 rats was fixed in Bouin's fluid, embedded in paraffin, and sectioned serially at 10 μ. The sections were stained with Harris' hematoxylin and eosin.

One adrenal from each rat was fixed for 48 hours in 10 per cent neutralized formalin, then washed in running tap water for an hour and sectioned on the freezing microtome at 15 μ. One section was stained with sudan IV and Harris' hematoxylin, one with sudan black B, and one by the Schiff "plasmal" method; these were mounted in glycerin jelly. Two other sections were mounted unstained in glycerin, one of which was untreated and the other extracted for one-half hour in acetone at room temperature. These two sections were compared under polarizing and fluorescence microscopes for acetone-soluble droplets showing birefringence and a greenish-white autofluorescence. The group of reactions employed characterizes the lipid droplets of steroid-secreting glands, and variations in reactivity have been correlated with different degrees of functional activity (Dempsey and Wislocki, 1946; Dempsey, 1948).

Another adrenal from each group of 3 rats was fixed in an alcoholic-acid silver nitrate solution for the demonstration of ascorbic acid (Barnett and Bourne, 1940). After fixation for one-half hour it was transferred to an acidified hypo solution for an hour (Deane and Morse, 1948). Then it was washed in running water, embedded in paraffin, and sectioned at 5 μ. The nuclei were stained with paracarmine.

2) *Sugar metabolism.* Blood-sugar levels were recorded during a period of complete starvation in 5 normal rats, in 10 rats whose adrenals had been enucleated for 1 month, and in 5 rats that had been adrenalectomized 1 week

previously and supplied with a 1 per cent sodium chloride solution for drinking water. Blood samples were taken from the tail by snipping off the tip. Samples were collected at 8 A.M. at the beginning of the fast and thereafter 12, 24, 36, 48, 72, 96 and 120 hours later. During the period of starvation the animals were kept in cages with wire mesh bottoms and were supplied with tap water, or 1 per cent sodium chloride solution in the case of the adrenalectomized rats, *ad libitum*. The adrenalectomized rats succumbed in 3 to 4 days, the others were still alive at the end of 5 days.

The level of sugar in the blood was determined by the modification of Folin's micro-method given in the Rubicon Co. manual for the Evelyn Photoelectric Colorimeter. The readings were made on an Evelyn Colorimeter.

3) *Injection of desoxycorticosterone acetate.* Four rats which had their adrenals enucleated for 1½ months received daily injections for a month of 2 mg. desoxycorticosterone acetate (Percorten, Ciba[3]) in oil. As controls, 2 untreated rats with demedullated glands were killed at the beginning and 2 at the end of the injection period. The adrenals were fixed in formalin and stained by the group of methods for indicating the presence of ketosteroids given above.

OBSERVATIONS

Histology and cytochemistry of the regenerating adrenal cortex

1 day. Twenty-four hours after enucleation, the adrenal gland appeared of normal size and shape both grossly and in section. The capsule was somewhat edematous. Beneath a large portion of the capsule no cortical cells could be found (Figs. 2 and 7), but in some places a few rows of cells remained, sometimes as many as 6 or 7 (Figs. 1 and 7). Only occasional abnormal mitotic figures in cortical

[3] The Percorten was generously supplied by the Ciba Pharmaceutical Products, Inc., Summit, N. J.

PLATE 1. Description of Figures.

All photomicrographs on this plate are from enucleated adrenal glands which were fixed in Bouin's fluid, embedded in paraffin, sectioned serially at 10 μ, and stained with hematoxylin and eosin. Photographed with green and yellow filters combined.

FIG. 1. Portion of adrenal enucleated for 1 day. At this site, about 5 rows of cortical cells remain attached to the capsule. The central part of the gland is filled with a blood clot and considerable accumulations of polymorphonuclear leucocytes. ×200.

FIG. 2. Another portion of same adrenal gland shown in Fig. 1. Here no parenchymal cells remain attached to the capsule. Compare with Fig. 7. ×200.

FIG. 3. Portion of adrenal gland enucleated for 3 days. The capsule appears edematous and its cells enlarged. The cortical cells now occur all around the gland and have formed short cords. M, mitotic figure in parenchymal cell. The central clot has become fibrous. ×200.

FIG. 4. Portion of adrenal gland enucleated for 8 days. The capsule still appears edematous. The cords of cells have lengthened somewhat. Mitotic figures (M) and recently divided cells (D) occur throughout. Connective tissue is developing in the center. ×200.

FIG. 5. Portion of adrenal gland enucleated for 18 days. The capsule has returned to essentially normal appearance. The cords have elongated considerably but often form whorls (compare Fig. 10). Mitoses (M) and recently divided cells (D) prevalent. ×200.

FIG. 6. Portion of adrenal gland enucleated for 32 days. The gland now possesses its normal architecture—narrow outer zona glomerulosa, a broad middle zona fasciculata, and an internal zona reticularis. No mitotic figures were found in this gland. ×160.

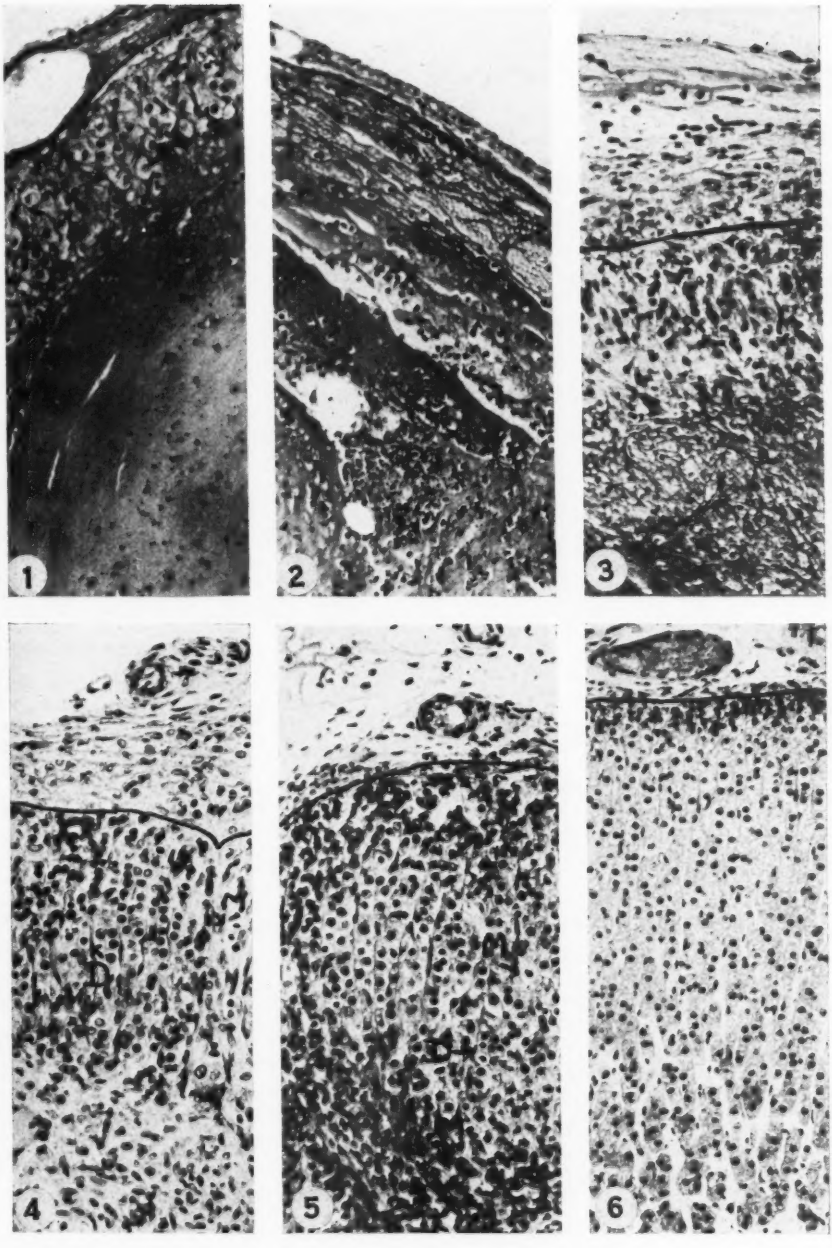
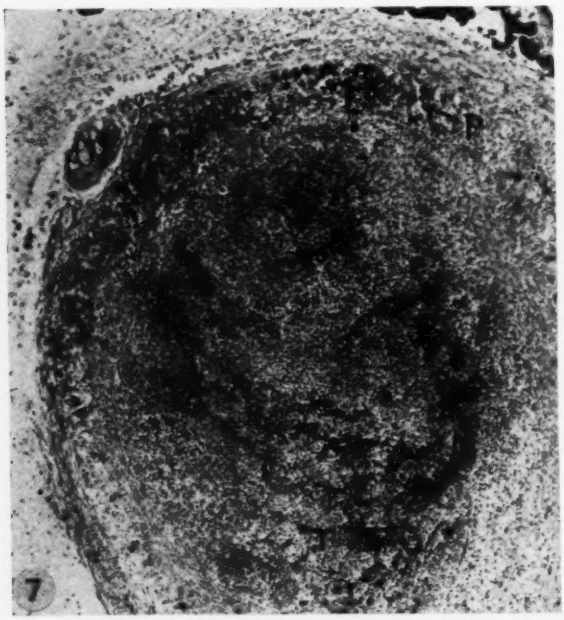


PLATE 1



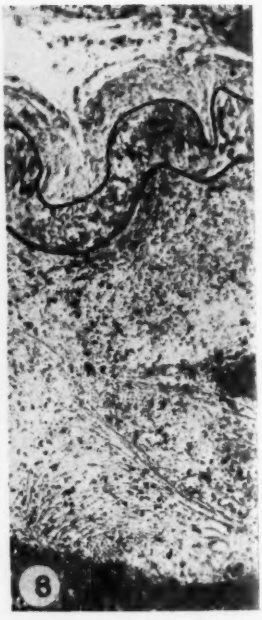


PLATE 2

cells were observed at this time (Deane and Morse, 1948, Fig 9). The central area was filled with a blood clot containing cellular debris, hemosiderin and numerous polymorphonuclear leucocytes. No loose parenchymal cells could be identified with certainty in the central clot.

One day after enucleation the few remaining parenchymal cells all contained a few sudanophilic droplets (Fig. 7) which were also Schiff-positive, autofluorescent and birefringent. The droplets were large and the birefringent crystals coarse. The cells lacked granules of reduced silver (Deane and Morse, Fig. 9).

3 days. Three days after enucleation the central clot was partially resorbed, causing the gland to appear collapsed and the capsule scalloped (Fig. 8). The capsule appeared more edematous than before and its cells, particularly those in its inner layer, were swollen and vacuolated (Figs. 3 and 8). No mitotic figures were found in capsular cells. The blood vessels in the capsule were distended with blood, suggesting that circulation had been restored. Within the central clot, leucocytes appeared degenerate and vacuolated (Fig. 8), and connective-tissue cells were making their appearance.

Cortical strands 8 to 12 cells in length extended down from the capsule all around the section. The cytoplasm of these cells varied in stainability—some cells stained with eosin, some failed to stain. Occasional mitotic figures were found, generally at about the midpoint of the cell columns (Fig. 3).

Only some of the parenchymal cells contained lipid droplets at 3 days (Fig. 8); these droplets exhibited the other lipid reactions employed as well, the birefringence being coarse. No ascorbic acid occurred in any cortical cells. Macrophages and degenerating leucocytes in the central area also contained sudanophilic droplets, but these

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PLATE 2

All photographs on this plate are of enucleated adrenal glands which were fixed in 10 per cent neutralized formalin for over 48 hours, washed, and sectioned on the freezing microtome at 15 μ. ×90.

FIG. 7. Adrenal gland enucleated for 1 day. Sudan IV and hematoxylin. Under the capsule fatty cortical cells (P) occur at some points, are absent elsewhere (compare Figs. 1 and 2). The dark material in the central clot is not fat but blood pigment. Periadrenal fat above.

FIG. 8. Segment of adrenal gland enucleated for 3 days. Sudan IV and hematoxylin. The gland has collapsed somewhat and the surface appears scalloped. The cortical cells (P) contain little lipid material. Macrophages and masses of degenerating blood cells in the internal scar contain considerable fat.

FIG. 9. Segment of adrenal gland enucleated for 8 days. Sudan IV and hematoxylin. Whorl of cortical cells above, mass of degenerating white blood cells below. Of the cortical cells, only the outermost ones contain any lipid.

FIG. 10. Segment of adrenal gland enuclated for 18 days. Schiff "plasmal" reaction. The cortical tissue occurs in whorls around the central scar. The cortical cells all contain droplets exhibiting the Schiff reaction.

FIG. 11. Segment of adrenal gland enucleated for 32 days. Sudan IV and hematoxylin. The parenchyma has regenerated to approximately normal dimensions and the cells all contain fatty droplets. Periadrenal fat above.

failed to display the other reactions and were probably triglycerides.

8 days. The capsule was still convoluted and appeared less edematous than at 3 days, although the connective-tissue cells remained swollen. The enlarged condition of these cells is clearly shown in the high-power drawings presented by Deane and Morse (Figs. 9 and 10). The central area was occupied by degenerating leucocytes, macrophages filled with hemosiderin, and numerous fibroblasts (Figs. 4 and 9). Distended blood vessels were prominent in the central scar, giving further evidence for the restoration of circulation.

The cortical tissue extended further inward from the capsule, some times in whorls (Fig. 9), sometimes in parallel columns arising from arcs underneath the capsule. The fascicles varied in length from 12 to 30 cells, averaging nearly 20. Mitotic figures were common and were distributed along the whole length of the fascicles. Even more common were pairs of cells with dense nuclei characteristic of recent division (Fig. 4).

At 8 days after enucleation small lipid droplets, displaying all of the ketosteroid reactions, were crowded in those cortical cells immediately below the capsule; below this narrow zone the cells lacked fatty material (Fig. 9). The birefringent crystals within the droplets were now fine rather than coarse. Likewise, sparse granules of ascorbic acid were present in the outermost row of cells (Deane and Morse, Fig. 10) but lacking below. Many cells within the scar appeared to be undergoing fatty degeneration (Fig. 9).

18 days. The capsule had now shrunken to approximately normal thickness (Fig. 5). The fibroblasts appeared elongate as though under tension. The central area was occupied by compact fibrous tissue. Histiocytes containing blood pigments occurred both in the capsule and in the central scar.

The parenchyma was arranged either in whorls, forming small separate glands, or in long fascicles (Figs. 5 and 10). No reticularis could be distinguished. All phases of cell division occurred along the length of the columns from the capsule to the scar border. Although elaborate counts were not undertaken, mitoses appeared to be more abundant at the 18-day stage than at any other studied.

Lipid droplets reacting positively to all of the tests used occurred throughout the regenerated cortical tissue. In some specimens the lipid droplets seemed very numerous in the outer portion of the gland and less so centrally; in others, all of the parenchymal cells were moderately laden (Fig. 10). Intracellular granules of ascorbic acid now entended far down the cortical strands, although they were sparse near the central scar.

32 days. The capsule appeared essentially normal in thickness and structure (Fig. 6). The central region was occupied by a small amount of scar tissue containing well developed veins.

The parenchyma was arranged in several major whorls (Fig. 11).

Within the individual whorls extended columns of parenchymal cells having the characteristics of normal cortex (Fig. 6). The outermost zone consisted of arcs 3 to 4 cells in width; the intermediate area comprised long fascicles about 25 cells long; centrally a broad reticularis, with wide sinusoidal spaces between the distorted fascicles, had developed. The cells in this zone did not appear degenerate. No mitotic figures were observed at this date.

All of the cortical cells contained numerous lipid droplets by the end of a month (Fig. 11). The birefringent particles were predominantly fine. Ascorbic acid had reappeared in all of the cortical cells in essentially normal amounts (Deane and Morse, Figs. 11 and 12). Fatty cells had entirely disappeared from the central scar.

To summarize these histological and cytochemical observations, a new cortex developed rapidly after adrenal enucleation, arising apparently from the residual glomerulosal cells. Mitosis occurred throughout the regenerating tissue, from immediately below the capsule to the inner border. Regeneration of an essentially normal cortex was complete by the end of a month. The capsular cells became enlarged and vacuolated in the early stages of regeneration, but no evidence of their division or transformation into cortical cells was found. After the operation a few large lipid droplets giving reactions characteristic of ketosteroids remained in the residual cortical cells but soon disappeared. No ascorbic acid was present in the cells at 1 or 3 days. A few small lipid droplets and granules of ascorbic acid made their appearance in the outermost cells of the regenerating tissue at the end of a week. Thereafter the amount of reactive material increased and spread down the regenerating fascicles, reaching normal concentrations by the end of a month.

Sugar metabolism

Experimental evidence indicates that the cells which secrete the "sugar" factors (the 11-oxygenated corticosteroids) lie in the zona fasciculata (Bergner and Deane, 1948). It was of interest, therefore, to determine whether or not rats with regenerated adrenals could secrete such hormones, especially since the regenerate seemed to be derived from cells originally lying in the zona glomerulosa. Normal rats, when starved, show a fall, then a rise in the blood sugar level (Long, Katzin and Fry, 1940); adrenalectomized rats show an almost continuous fall. The changes in blood-sugar level of rats whose adrenals had regenerated for a month were therefore compared during a fast with those of intact and adrenalectomized rats.

During the period of starvation, the intact rats lost 19 per cent of their weight in 4 days, 26 per cent in 5 days; the animals with regenerated adrenals lost 15 per cent in 4 days, 19 per cent in 5 days; and the adrenalectomized rats lost 14 per cent in 4 days.

Figure 12 records the changes in the level of blood sugar observed

in the 3 groups. The rats with demedullated adrenals behaved similarly to the intact animals. The level of blood sugar dropped for 36 to 48 hours, then rose to the original level. The adrenalectomized animals began with a somewhat lower blood sugar, and the level fell steadily until coma and death ensued at 3 to 4 days.

Injection of desoxycorticosterone acetate

Since the structure and cytochemical appearance of the adrenal cortex were restored to normal a month after enucleation, it was of

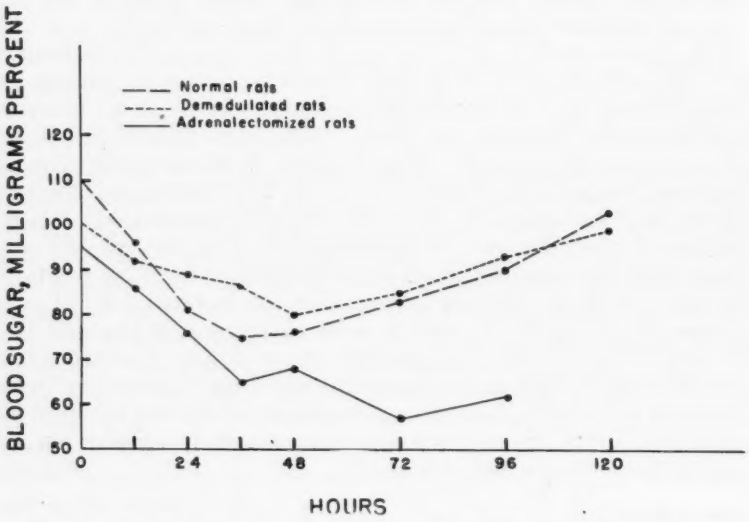


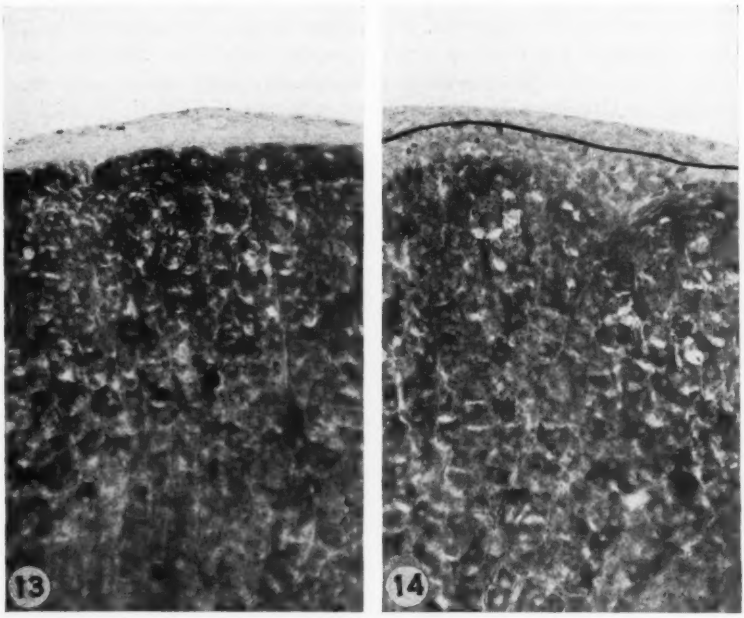
FIG. 12. Changes in blood-sugar levels in groups of normal rats, rats with regenerated adrenal glands, and adrenalectomized rats during a period of complete starvation.

interest to learn if the normal functional zonation was restored as well. In normal animals the injection of desoxycorticosterone acetate appears to cause a suppression of secretory activity in the zona glomerulosa (Greep and Deane, 1947), a finding which suggests that the salt-retaining hormones are secreted by this zone.

Four rats whose adrenals had regenerated for 1½ months were injected daily for a month with 2 mg. desoxycorticosterone acetate, then killed. Their adrenals were compared with the adrenals of 2 animals with demedullated glands killed at the beginning of the injection period and 2 others killed at the end.

The regenerated adrenals from animals without hormone treatment presented the normal appearance, with a large amount of lipid material displaying the various lipid reactions present in the cells of

the zona glomerulosa and somewhat less in the cells of the zona fasciculata (Fig. 13). The adrenals from the rats treated with desoxycorticosterone acetate, however, showed shrinkage of the cells of the glomerulosa and a complete disappearance of lipid droplets therefrom (Fig. 14). The fasciculata appeared unaffected.



Photomicrographs of adrenal glands enucleated for 2½ months. Sudan IV and hematoxylin. ×200.

FIG. 13. Gland of an untreated animal. Sudanophilic droplets present in both the glomerulosa and the outer part of the fasciculata.

FIG. 14. Gland of a rat injected for last month with 2 mg. desoxycorticosterone acetate daily. The cells in the outermost zone, the glomerulosa, are shrunken and entirely depleted of lipid droplets. Lipid remains in the cells of the fasciculata.

DISCUSSION

Regeneration of the cortex after enucleation of the adrenal

On the basis of a variety of experimental procedures, several investigators have maintained that, in the rat, new parenchymal cells of the adrenal cortex develop directly from fibroblasts in the capsule (Zwemer, Wotton and Norkus, 1938; Baker and Baillif, 1939; Salmon and Zwemer, 1941; Wotton and Zwemer, 1943; Baxter, 1946; reviewed by Jones, 1948). Gruenwald and Konikov (1944), who support this thesis with respect to many other species, have failed to detect signs of conversion of capsular cells into cortical cells in the

adrenals of rats. In the present experiments, employing a situation in which the demand for new cortical cells was very high, no signs of such conversion were seen. In the immediate post-operative period the capsule became edematous so that the fibroblasts appeared rounded. Nevertheless, the difference between fibroblasts and cortical cells remained distinct at all times. Furthermore, no mitoses were observed in the capsular cells, whereas mitoses were prevalent among the cortical cells. Since patches of cortical tissue invariably remained attached to some portions of the capsule, even after the attempt to strip it completely, proliferation of these cells seems most probably to account for all of the new parenchyma.

For the time being it seems reasonable to assume that the residual cortical cells spread out rapidly after the operation to line completely the inner border of the capsule and thus provide the "seed" for the regenerate. This conclusion agrees with the observations of Williams (1947), who obtained no indisputable evidence for the change of capsular into cortical cells in regenerating adrenal grafts. More recently, Everett (1949) has demonstrated proliferation of cortical cells when transplanted beneath the kidney capsule. Inclusion of the adrenal capsule was not essential.

During the regenerative process, cell division occurred along the entire length of the growing fascicles, rather than being limited to an outer zone. This distribution of mitotic figures suggests centripetal proliferation of the cells (Vaccarezza, 1945–6; Mitchell, 1948) rather than migration of cells from the capsular region. Nevertheless, it is an inescapable conclusion that cells which ultimately came to lie in the inner zones arose from glomerulosal cells. In an earlier paper (Deane and Greep, 1946) we stated that, on the basis of the independent reactions of the glomerulosa and fasciculata to hypophysectomy, "many facts make it seem probable that the two zones are separable and that very few cells of the glomerulosa actually migrate into the fasciculata." Apparently, under extreme circumstances at least, this conversion may occur. What proportion of the inner tissue normally derives from the glomerulosa can not be ascertained by histological techniques at present available. The use of colchicine, for instance, to determine the number of cell divisions in each zone imposes severe limitations when employed to analyze growth of cortical tissue, since this drug itself constitutes a physiological stress (Selye, 1946). Thus it undoubtedly stimulates the fasciculata at the same time that it arrests cell division, a conclusion reached by Baxter from the study of animals with and without colchicine treatment.

Restoration of secretory activity in the regenerating cortex

Two cytochemical methods were employed to indicate the secretory activity of the cortical cells. One involved the use of frozen sections prepared by a variety of methods for characterizing the lipid

droplets. Past experience has shown that the parenchymal cells in organs known to secrete steroid hormones contain droplets that are sudanophilic, Schiff-positive and autofluorescent and contain birefringent crystals (Dempsey and Wislocki, 1946; Dempsey, 1948). Whatever the actual constitution of such droplets, their presence, number and size have proved valuable in ascertaining the physiological status of the adrenal cortex (Deane, Shaw and Greep, 1948). The other method involved fixing the organ in acidified silver nitrate to reveal reducing activity presumably limited to ascorbic acid (Deane and Morse, 1948). Ascorbic acid has been shown to be especially concentrated in those organs which secrete steroid hormones, although it is not limited to them (Giroud and Leblond, 1935).

Immediately following enucleation of the adrenal, ascorbic acid disappeared entirely from the residual cortical cells. Some large lipid droplets containing coarse birefringent particles remained, however. Droplets of this character appear to signify that hormone is not being released (Deane, Shaw and Greep). Only when regeneration had proceeded for about a week did granules of ascorbic acid and small lipid droplets with fine birefringence make their reappearance. At first both were limited to the cells immediately underlying the capsule; gradually, during the succeeding 2 to 3 weeks, they reappeared in all of the new cortical tissue. The reappearance of these two types of material indicates that hormone secretion is reestablished at least within a week after enucleation, a conclusion supported in part by the observation that rats with demedullated adrenals do not require additional sodium chloride to survive, as do adrenalectomized animals (Ingle and Higgins, 1938). Indeed, Marenzi (1938) found no disturbance whatsoever in the electrolyte balance of demedullated animals.

Zonation of the regenerated gland

In view of the major part in the regeneration of the cortex played by cells originally lying in the zona glomerulosa, the possibility existed that the regenerate would function solely as glomerulosa, i.e., would secrete hormones governing the metabolism of electrolytes but not of proteins and carbohydrates. To test this hypothesis, we determined the ability of animals with newly regenerated cortices to maintain the blood sugar at normal or near normal levels during complete starvation. It is well known that in adrenalectomized rats the blood sugar level declines rapidly during fasting, leading to early death. Fasting, intact rats, on the other hand, show an initial decline in blood sugar, then a return to normal or supra-normal values (Long, Katzin and Fry, 1940). This secondary increase in the blood sugar is believed to depend on gluconeogenesis induced by 11-oxygenated corticosteroids (Ingle and Thorn, 1941; Olson, Thayer and Kopp, 1944). The response may be considered one example of the adaptation of the animal to a physiological stress (Selye).

One month after enucleation of the adrenals, rats responded to starvation in the same way as intact controls. While adrenalectomized animals died in hypoglycemic coma on the 3rd or 4th day, the normal rats and rats with regenerated adrenals survived more than 5 days and their blood sugar levels returned to 100 mg. per cent or more. This test leaves no doubt as to the ability of the regenerated cortex to influence protein and carbohydrate metabolism. Hence it is concluded that cortical cells derived from the glomerulosa can secrete 11-oxygenated hormones.

Other investigators have also found that animals with regenerated adrenals give evidence of secreting 11-oxygenated corticosteroids. Evans (1936), one of the original investigators to employ the technique of demedullation of the adrenal, reported that rats, 10 to 18 days after operation, were intermediate between intact and adrenalectomized animals in their capacity to deposit glycogen in the liver when subjected to stress. Brownell and Hartman (1948) obtained signs of unusually high secretion of cortical hormones (apparently 11-oxygenated) influencing both fat and sugar metabolism within 9 and 13 days, respectively, after adrenal enucleation. Furthermore, Ingle, Li and Evans (1946) observed glycosuria, increased blood glucose, and increased urinary non-protein nitrogen in 2 of the 3 rats given adrenocorticotropin a considerable period after demedullation of the adrenals. The remarkable thing is that we observed a normal blood-sugar response in all 10 of the rats tested, despite more severe stripping of the capsule than employed by Ingle.

Not only is the capacity of the cortical tissue to secrete both types of hormone restored upon regeneration, as indicated by the failure of rats to require extra sodium chloride and by the normal "sugar" response, but apparently the normal zonation of secretory activity is restored. In rats with regenerated adrenals, as in animals with intact glands, the administration of desoxycorticosterone acetate over a period of a month causes shrinkage of the glomerulosal cells and the disappearance of all lipid droplets. No significant alteration occurs in the inner zones. These changes in the glomerulosa have been interpreted as a "disuse atrophy" resulting from the exogenous supply of a hormone capable of substituting for the normal product of the zone (Greep and Deane, 1947). The response provides presumptive evidence that the outer rim of the cortex alone is responsible for secreting the "salt" factor in the regenerated adrenal as in the normal gland.

These observations on the restoration of functional zonation raise again the question of the basis for that zonation. Apparently cortical cells originating from the peripheral region, whether in normal organogenesis or after enucleation of the gland, have different functions at various levels from the surface. Is the formation of 11-oxygenated hormones dependent upon the presence of desoxycorticosterones in

the blood bathing the cells? Does the formation of the desoxycorticosterone-like hormones require more oxygen, more food stuffs and fewer waste products than the formation of 11- oxygenated hormones? Or, adopting either the thesis of cell migration or that of centripetal proliferation, does the age of the cell in some way influence the nature of its product? The functional zonation of the liver lobule, while less definite than that of the adrenal cortex, has compelled appreciation of the rôle of the blood supply in influencing cellular activity (Deane, 1944; Himsworth, 1948). In many ways the blood supply of the liver lobule and of the adrenal cortex is similar. More consideration of this provocative problem might prove rewarding in the understanding of the underlying biology of secretion.

SUMMARY

Following enucleation of both adrenal glands in the adult rat, proliferation of the residual cortical tissue (glomerulosal cells) restores a normal appearing cortex within a month. The regenerated cortex possesses the usual zona glomerulosa, zona fasciculata and zona reticularis.

No evidence has been found for the conversion of capsular fibroblasts to cortical cells.

Within 8 days after the operation, ascorbic acid and droplets giving the reactions characteristic of steroid-producing glands reappear in the immediate sub-capsular cells and eventually come to occupy all of the cells of the cortex.

One month after enucleation of their adrenals, rats respond in the normal fashion to a prolonged fast, i.e., the blood-sugar level declines temporarily, then returns to 100 mg. per cent within 5 days. Adrenalectomized rats succumb within 4 days in hypoglycemic coma.

The daily administration of desoxycorticosterone acetate over a period of a month causes a suppression of secretory activity in the zona glomerulosa of the regenerated gland.

The regenerated cortex apparently secretes hormones governing both salt and sugar metabolism, and the normal functional zonation is restored.

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CHANGES IN SERUM CHOLESTEROL AND CHOLESTEROL ESTERS IN ALLOXAN DIABETIC RABBITS

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THE EFFECT of diabetes mellitus on the ratio of free to esterified cholesterol of the serum has not been established (Peters and Van Slyke, 1948) although it is generally recognized that disturbances of cholesterol metabolism do occur in this condition. The usual manifestation is a hypercholesterolemia which does not seem to be related to hyperglycemia, glycosuria, or ketonuria although it is more marked in severe cases of diabetes with a tendency to ketosis and acidosis. In patients controlled either with diet or with insulin, there is usually a normal concentration of serum cholesterol; this is not invariably true as there are several reports of diabetic subjects who continued to have high postabsorptive serum cholesterol levels, even when their diabetes was well controlled (Peters and Van Slyke, 1948).

It is difficult to interpret the cause and effect relationship of cholesterol metabolism to clinical diabetes because of the complicated picture of human diabetes. Accordingly, Chaikoff and Kaplan (1933) reported a study of serum cholesterol changes in depancreatized dogs maintained on insulin wherein they showed that the cholesterol ester fraction practically disappeared while the free cholesterol fraction was only slightly lower than that of normal control animals. However removal of the pancreas imposes a multiple deficiency and it was show by Gibbs and Chaikoff (1941) that the diet of the depancreatized dogs as well as the administration of insulin modified the level of serum cholesterol and the ester fraction.

With the discovery of Dunn, Sheehan, and McLetchie (1943) that the administration of alloxan to animals would cause the diabetic condition, a new approach for the study of the relationship of cholesterol metabolism to diabetes was opened. Moreover this procedure has the advantage of producing an essentially uncomplicated diabetic state in animals (Lukens, 1948). However, studies along this line are lacking with the exception of the report by Kendall, Meyer, Lewis, and Victor (1945) that following the injection of alloxan into rabbits

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there developed a transient period of hypercholesterolemia which accompanied the early stages of severe diabetes. This report dealt only with the changes in total cholesterol of serum, omitting any reference to the partition of free and ester cholesterol. A further study of the effect of alloxan diabetes on the changes in serum cholesterol and cholesterol esters in rabbits is reported in this paper.

EXPERIMENTAL

Six albino rabbits, each weighing about 2 kilograms, were used. The rabbits were kept on a diet of Purina Rabbit Chow and water ad lib. During a control period of two weeks, the rabbits were bled seven times; the bloods were analyzed for sugar by the micro method of Folin and Malmros (1929) and for free and total cholesterol by a modification of the method of Schoenheimer and Sperry as described by Hawk, Oser, and Summerson (1947). Samples of approximately 1.0–1.5 cc. of blood were taken both when the animals were fasted and when food was allowed in the cage overnight; inasmuch as the control values were the same under both conditions, all control blood levels of sugar and cholesterol were grouped together (Table 1).

TABLE 1. SERUM CHOLESTEROL AND BLOOD GLUCOSE LEVELS DURING CONTROL PERIOD. EACH VALUE REPRESENTS THE MEAN OF SEVEN DETERMINATIONS OVER A PERIOD OF TWO WEEKS

Rabbit	Serum cholesterol				Blood glucose mg. %
	Total mg. %	Free mg. %	Ester mg. %	Ratio of ester to total as %	
1	56.1 ± 2.20*	23.5 ± 0.91	32.6 ± 1.89	57.9 ± 1.64	111.1 ± 2.38
2	83.4 ± 1.68	38.0 ± 1.99	45.4 ± 2.01	54.4 ± 1.55	98.1 ± 2.72
3	46.4 ± 2.36	19.9 ± 1.17	26.5 ± 1.55	57.2 ± 1.48	106.0 ± 3.67
4	64.6 ± 5.60	26.8 ± 1.92	37.8 ± 3.82	58.0 ± 1.88	101.3 ± 3.36
5	53.7 ± 1.96	23.1 ± 1.31	30.6 ± 1.57	57.1 ± 1.74	109.4 ± 2.78
6	50.2 ± 1.92	21.9 ± 0.40	28.3 ± 1.64	56.2 ± 1.26	109.5 ± 1.64
Mean	59.1 ± 2.62	25.5 ± 1.28	33.5 ± 2.08	56.8 ± 1.59	105.9 ± 2.76

* Represents the standard error of the mean calculated by the formula,

$$\sqrt{\frac{\sum d^2}{n-1}} \div \sqrt{n}$$

Without preliminary fasting, each rabbit received intravenously 200 mg. of alloxan monohydrate (Eastman Kodak Co.) per kilogram of body weight. This dose of alloxan was made up in 4 cc. of distilled water and was administered in two injections one half hour apart. Hypoglycemic convulsions developed in two animals (no. 4 and 5) in four and one half hours and were successfully controlled with intraperitoneal and subcutaneous injections of glucose solution. The four remaining animals were bled 4.5 hours after injection of alloxan and they too were given injections of glucose to prevent hypoglycemic

shock. Subcutaneous injections of glucose were continued for all animals for a period of twenty four hours. The animals were again bled twenty four hours later at which time they had access to food and water. Following this initial period of forty eight hours after the administration of alloxan, the animals were fasted overnight and samples of blood were again taken. This procedure was repeated daily for the first two weeks and thereafter at two day intervals. Thirty three days after the administration of alloxan, the rabbits were given daily morning injections of 2 units of protamine-zinc-insulin. This was increased every week by 2 units until a daily dose of 8 units was reached in five of the animals; rabbit number 2 died during the 6 unit period.

RESULTS

Within two or three days after the administration of the alloxan, all six animals exhibited hyperglycemia and in five of the six rabbits there was an increase in the total serum cholesterol. The total cholestrol continued to rise until a peak was reached between the seventh and tenth days; this peak value varied from 88.9 mg. per cent in rabbit 3 to 688 mg. per cent in rabbit 5. Thus the increases over the control values ranged from 92 per cent in rabbit 3 to 1170 per cent in rabbit 5. The serum cholesterol then began to decrease and had returned to the pre-alloxan level by the seventeenth day. The increase in total cholesterol was paralleled by an increase in the free cholesterol in all six animals; rabbit 6 in which there was no increase in total cholesterol exhibited a twofold increase in free cholesterol. These high levels varied from 40.0 mg. per cent in rabbit 6 to 525 mg. per cent in rabbit 5. The drop in free cholesterol to pre-alloxan values paralleled the decline in total cholesterol.

The changes observed in the ester cholesterol and the ester: total cholesterol ratio (expressed as per cent ester cholesterol) were in the opposite direction to the changes in the total and free cholesterol. While these latter values rose during the first week, the ester cholesterol remained the same in rabbits, 2, 3, fell sharply to 7.0 and 6.7 mg. per cent in rabbits 4 and 6, or showed a slight increase which lagged behind the increase of the free cholesterol fraction (rabbits 1, 5). Irrespective of the direction of change or lack of change in the ester fraction, there occurred a definite decrease in the ester to total ratio in all six animals. This ratio varied from a value of 3.3 per cent in rabbit 4 to 40 percent for rabbit 3 whereas the control ester: total ratios were in the vicinity of 56 to 58 per cent in all six animals (Table 1). In this first week after the injection of alloxan, five of the six animals had an ester: total ratio of less than 30 per cent while in three of the rabbits, the ratio dropped to less than 15 per cent. Following the marked drop in the ester: total cholesterol ratio, there was a return of the ratio values to control levels at about the twelfth post-alloxan day.

After the diabetes had assumed a steady state, as shown by the rather constant values for blood sugar and serum cholesterol fractions in the fasting animals from the twenty first to the thirty third days, the animals received daily injections of protamine-zinc-insulin. By the tenth day of insulin administration, there was a definite elevation of the total cholesterol levels in five of the six animals. The elevated levels varied from 63.0 mg. per cent in rabbit 3 to 240 mg. per cent in rabbit 5. Rabbit 6, which did not exhibit an increase in the total

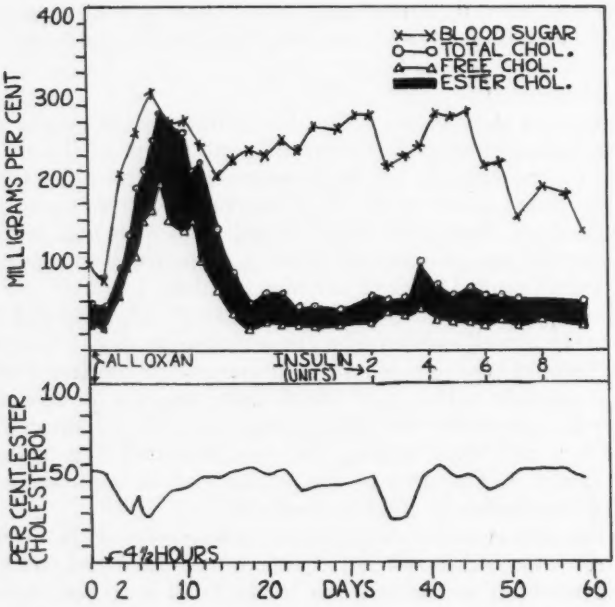

FIG. 1. Effects of alloxan and protamine-zinc-insulin on blood sugar, total serum cholesterol, free cholesterol, ester cholesterol, and ester: total cholesterol ratio. Each point represents the mean value for six rabbits.

cholesterol following alloxan administration, was the exception in this case. The increase in total cholesterol was paralleled by a comparable increase in the free cholesterol. The ester cholesterol and the ester: total cholesterol ratio dropped to extremely low levels in five of the six animals. The ester cholesterol decreased to 4.6, 5.5 and 4.6 mg. per cent respectively in rabbits 3, 4, and 6 during the first two to four days after the start of the insulin; rabbits 2 and 5 showed a moderate decrease to a level of 20 mg. per cent while in rabbit 1 there was no change in the level of the ester fraction. In all six animals there occurred a definite decrease in the ester: total ratio during the two to five day period after the institution of insulin therapy. The ester:

total cholesterol ratio varied from 5.4 per cent in rabbit 4 to 36.7 per cent in rabbit 1; in three of the six animals, the ratio was less than 20 per cent. Within six to eight days after the initiation of insulin injections, the cholesterol ester fraction and the ester: total ratio returned to the level of the control values. The results for all six animals are graphically presented in Figure 1. Rabbit 4 was deemed as being most representative of the group and the results for this animal are plotted in Figure 2.

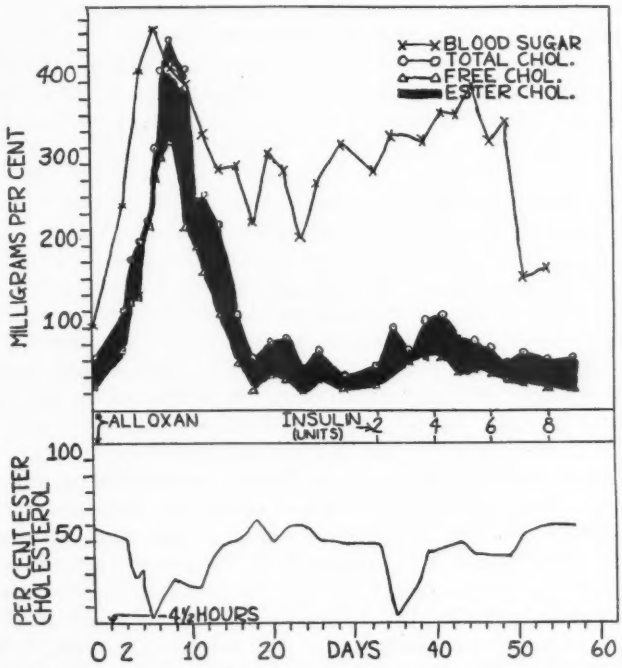

FIG. 2. Effects of alloxan and protamine-zinc-insulin on blood sugar, total serum cholesterol, free cholesterol, ester cholesterol, and ester: total cholesterol ratio in a representative rabbit (no. 4).

DISCUSSION

It is worth mentioning that with this method of administering alloxan in divided doses, a state of diabetes was produced in all six animals without a death from the usual toxic effects of alloxan (Lukens, 1948). To date we have administered alloxan according to this dosage schedule to sixteen rabbits in the course of this and other work; there have been no deaths and fifteen of the sixteen animals have been made diabetic.

The marked and sudden increase in the total cholesterol of the serum after the injection of alloxan is in agreement with the findings

of Kendall, Meyer, Lewis, and Victor (1945). Inspection of the data for the fractions of serum cholesterol shows that this hypercholesterolemia is at first due to a marked rise in the free cholesterol with the ester fraction decreasing or remaining fairly constant; this produces the marked decrease in the ester: total ratio. At six to eight days after the injection of alloxan, the increase in the total and free cholesterol has reached its peak. At this time, there is an absolute increase in the ester cholesterol and the ester: total ratio begins a return to control levels. Within twenty days after alloxan administration, all cholesterol values have returned to their pre-alloxan concentrations. There does not seem to be any relationship between the blood sugar and the serum cholesterol except for the fact that both increase within a few days after the injection of alloxan. The increase in serum cholesterol at this time, as suggested by Kendall, Meyer, Lewis, and Victor (1945), is probably due to the mobilization of lipids from the fat depots and body tissues. This is supported by the observation (Goldner and Gomori, 1943) that there is a fatty infiltration of the liver within ten to twelve days after alloxan is administered.

With the administration of insulin to the diabetic rabbits, there occurs an increase in the total and free serum cholesterol with a concomitant drop in the ester: total ratio. The changes are similar to those that occur during the first week of diabetes following the injection of alloxan. Since the increase in total serum cholesterol after the development of diabetes is thought to be due to the mobilization of cholesterol from the fat depots and other body tissues, it is suggested that subsequent to the daily injections of insulin there is a reversal of this process with the lipids decreasing in the liver and returning to the other body tissues. This is consistent with the knowledge that cholesterol in the body tissues exists as free cholesterol and not in the esterified form. Thus when the body tissues undergo a loss of cholesterol, this would be reflected in the serum as an increase in the free cholesterol. This in turn is measured as an increase in the total serum cholesterol and a decrease in the ester: total ratio. Following the increase in available free cholesterol, there is a rise in the absolute amount of serum ester cholesterol because of the presence of a cholesterol esterifying enzyme in the blood (Sperry and Stoyanoff, 1937, 1938) and in the liver (Klein, 1938; Sperry and Brand, 1941).

SUMMARY

There is an increase in the total cholesterol and free cholesterol of the serum following the injection of alloxan in rabbits. At the same time, there is a marked drop in the ratio of ester: total cholesterol of the serum, while the absolute amounts of serum ester cholesterol may decrease, or remain relatively stationary. Following the daily injection of protamine-zinc-insulin in alloxan diabetic rabbits, similar changes

in serum cholesterol and per cent of ester takes place. A possible mechanism underlying these changes is discussed.

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A SIMPLIFIED PROCEDURE FOR THYROIDECTOMY OF THE NEW-BORN RAT WITHOUT CONCOMITANT PARATHYROIDECTOMY[1]

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SURGICAL thyroidectomy of the very young rat is difficult because of the size of the animal and the condition of its tissues. The operation has been performed successfully, however, by two groups of workers. Salmon (1936), who first demonstrated the feasibility of surgical excision of the thyroid gland, along with the parathyroid, in this animal as early as one or two days after its birth, employed cold anesthesia and observed survival for longer than one week in about 36 per cent of the operated rats. Scow and Simpson (1945), who employed a similar technique, with even lower temperatures for anesthetic purposes, showed survival of less than seven per cent of their totally thyroidectomized rats. In view of these difficulties, we wish to report a simplified procedure for discrete total thyroidectomy of the new-born rat without recourse to the more complicated surgical technique. This has been accomplished by the use of radioactive iodine, which, as was to be expected, can be used to destroy thyroid tissue. The purpose of the present investigation is to establish a dose that achieves complete thyroidectomy by internal radiation of the gland in the 4–12-hour-old rat and which, in addition, is of sufficiently low intensity to produce either minimal or non-deleterious effects on extrathyroidal tissues.

EXPERIMENTAL

Pregnant rats of the Long-Evans strain were fed stock diet No. 2, the composition of which has been described elsewhere (Taurog and Chaikoff, 1946). Several hours after birth, two rats of each litter were injected intraperitoneally with varying doses of carrier-free, radioactive iodine and two (one male and one female) were retained as controls. Each litter was limited to four rats. The injected rats were kept with their mothers until weaning at 28 days of age. The controls remained with their mothers for one week and were then transferred to a foster-mother. This was found necessary because the in-

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[2] Atomic Energy Commission Fellow.

jected rats were unable to compete with their litter mate controls for their mother's milk. The injected rats received no special care other than that accorded to growing rats.

At various ages, the rats were anesthetized by an injection of 0.5 cc. of two per cent sodium pentobarbital, and exsanguinated. The entire neck region (soft tissues anterior to prevertebral fascia) was excised and fixed in Bouin's fluid. Representative portions of the various organs were also removed and fixed in a similar manner. The pituitary was fixed in a fluid composed of nine parts of a saturated solution of mercuric chloride and one part of 40 per cent formalin. All tissues were embedded in paraffin. The entire neck region was sectioned serially, and representative sections were prepared from the other organs. The pituitary glands were sectioned at 2–3μ, and all other tissues at 6μ. Mallory and haematoxylin and eosin stains were used for all tissues except the pituitary which was stained by the method of Martins (1933) as modified by Griesbach (personal communication). Pituitary cell counts were made after the method of Griesbach (1943); at least 2000 cells per gland were counted.

RESULTS

A total of 28 new-born rats from eight litters have been injected with radioactive iodine. Table 1 records typical observations made on the first five litters studied.

As judged by examination of serial sections of the entire neck region, no thyroid tissue was present in rats that had been injected with

TABLE 1. THE THYROIDS AND ANTERIOR PITUITARIES OF RATS INJECTED WITH VARIOUS DOSES OF I^{131}

Litter	Rat no.	I^{131} injected	Age sacrificed	Weight	Condition of thyroid	Anterior pituitary cell count		
						Chromophobes	Acidophils	Basophils
		μc.	weeks	grams		per cent of total	per cent of total	per cent of total
1	1	150	1†	5	Completely destroyed	80.5	3.1	16.4
	1a	None	1	11	Normal	—	—	—
	2	100	2	12	Completely destroyed	74.4	1.3	24.3
	2a	None	2	17	Normal	50.4	43.7	5.9
2	3	80	11	29	Completely destroyed	81.7	0.9	18.4
	3a	None	11	180	Normal	52.4	42.5	5.1
	6	80	19*	68	—	—	—	—
	6a	None	19*	289	—	—	—	—
3	4	80	4.5	26	Completely destroyed	78.9	1.0	20.1
	4a	None	4.5	87	Normal	49.3	44.5	6.2
	7	80	11*	32	—	—	—	—
	7a	None	11*	184	—	—	—	—
4	5	60	4.5	36	Some follicles with colloid, abnormal	55.6	24.3	20.1
	5a	None	4.5	86	Normal	50.3	44.4	5.3
	8	60	9	68	Small, normal	67.0	14.7	18.3
	8a	None	9	204	Normal	54.2	40.9	5.9
5	9	40	7	84	Normal and small	65.5	15.4	19.1
	9a	None	7	143	Normal	47.4	47.5	5.1
	10	40	11*	112	—	—	—	—
	10a	None	11*	192	—	—	—	—

* Not sacrificed.
† Died from inanition (see text).

amounts of I^{131} varying from 80 to 150μc. In these rats, the region formerly occupied by the thyroid was replaced by adipose and collaginous tissue. Except for a single injected rat (Table 1, No. 3) which developed diarrhea at the age of seven weeks, all I^{131}-treated rats were in good physical condition until sacrificed. Rat No. 1 died at six days of age, probably as a result of inanition caused by inability to compete with its two normal litter mates for its mother's milk. This rat belonged to the first litter studied and its death from malnutrition led us to segregate the injected and control rats in later experiments as described above.

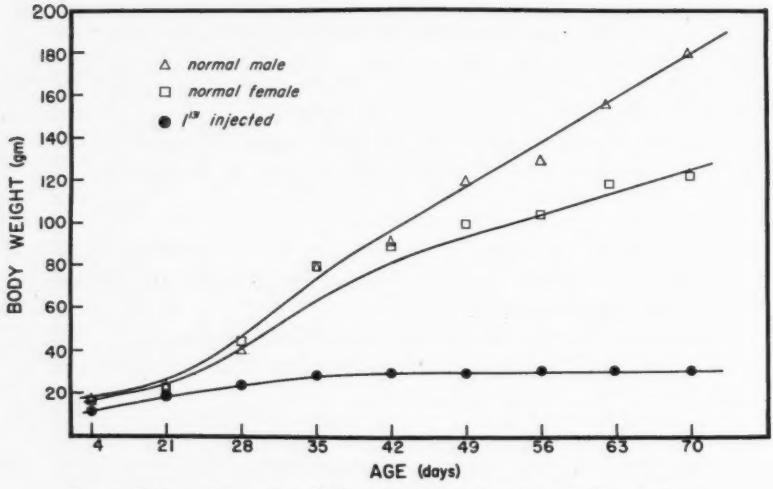

FIG. 1. Body weights of radiothyroidectomized and normal control rats.

A comparison of the growth response of rats that had been injected with 80μc of I^{131} with that of their untreated litter mate controls (male and female) is shown in figure 1. The data were obtained from litters 2 and 3 (Table 1) and each point represents the average of four measurements on as many rats. At the age of 75 days, the highest weight attained by the I^{131}-treated rats was 32 gm.; at this age, the normal male rat weighed 180 gm. (fig. 3). The oldest injected rat so far examined weighs 68 gm. at 19 weeks of age; its litter mate male control weighs 289 gm.

The roentgenogram of rat No. 3 (fig. 2), taken at the age of 75 days, clearly demonstrates the retardation of bone growth. This rat had been injected with 80μc of I^{131}. Its skeletal age, as judged roentgenographically, is that of a normal, 18 to 20-day-old rat. The criteria for estimating the bone age of this strain of rat were those employed by Scow and Simpson (1945), and Becks *et al.* (1948).

FIG. 2. Roentgenogram of a 75-day-old rat injected at birth with 80 μc of I^{131}. The skeletal age of this rat is 18–20 days.

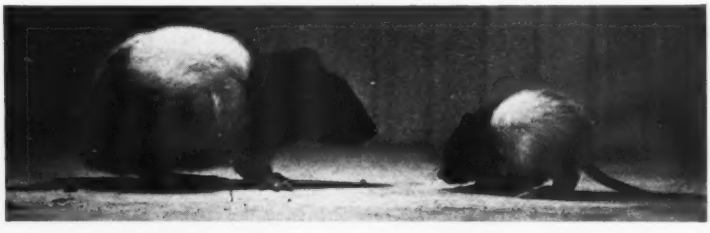

FIG. 3. Litter mate male rats, 75 days old. The smaller was injected with 80 μc of I^{131} a few hours after birth and weighs 32 gm. Its normal litter mate weighs 180 gm.

Minute amounts of thyroid tissue were found in rats that had been injected with 60 and 40μc (Table 1). In those that had received 60μc, well defined follicles with typical epithelium were found. In one rat which had received 60μc of I^{131} (No. 5), an atypical thyroid proliferation was observed, consisting of a unilateral, partially unencapsulated mass of epithelial cells with a fetal type of architecture. The isthmus and the other lobe consisted of typical thyroid epithelium with well defined, but small, follicles which were filled with pale, eosinophilic colloid.

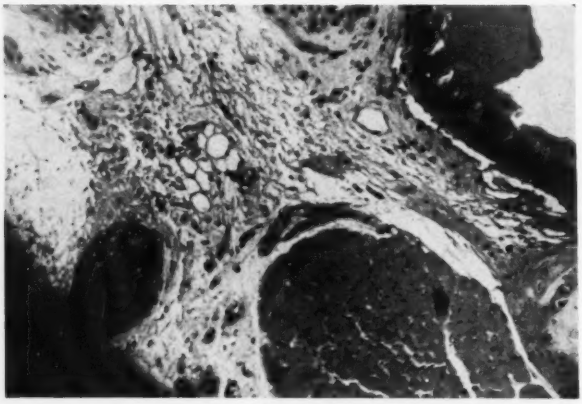

FIG. 4. Longitudinal section of the trachea of a rat that had been injected with 80 μc of I^{131}. Note in the lower left corner the intact parathyroid gland surrounded by adipose and collagenous tissue. Tracheal epithelium is shown in upper right hand corner. (Haematoxylin and eosin; 10 μ; $\times$125.)

The pituitary changes found in the I^{131}-treated rats are recorded in Table 1. The acidophils were almost completely degranulated in rats in which no thyroid tissue was detected by histological examination of serial sections of the neck region (rats Nos. 2, 3, and 4). Interestingly enough, in rats in which as few as 14–16 surviving follicles remained, degranulation of the acidophils was only slight. The presence of typical thyroidectomy cells (large, vacuolated basophils) were observed in all I^{131}-treated rats; this finding indicates that even the lowest dose of I^{131} injected had resulted in sufficient thyroid damage to induce a thyroxine deficiency.

No pathological changes were observed in liver, kidney, gastrointestinal tract, and heart. Evidence of damage (loss of ciliation and nuclear pyknosis) were found in the tracheal epithelium in the region adjacent to the destroyed thyroid gland. A few pyknotic and some atypical enlarged hyperchrorratic chondrocytes were also found in the adjacent cartilage.

Intact parathyroids were found in all rats in which complete de-

struction of the thyroid gland had occurred (figs. 4 and 5). Aside from a few pyknotic cells in the peripheral portions, and some increase in interstitial connective tissue and a thickened capsule, the parathyroids appeared essentially normal histologically. No tetany was observed in any rats.

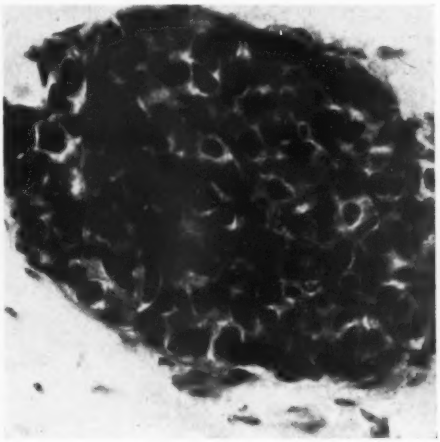

FIG. 5. Parathyroid gland shown in fig. 4, ×500.

COMMENT

No single criterion can be used to establish completeness of thyroidectomy. For this reason, evidence as to whether the thyroid gland had been completely destroyed in new-born rats by the injections of I^{131} was sought by a study of growth response, bone age, and anterior pituitary cytology in addition to histological examination of serial sections of the entire neck region. As judged by these criteria, it would appear that total thyroid destruction can be achieved in the newborn rat by the injection of 80 or more μc of I^{131}.

Griesbach (1943, 1945, and 1946) has called attention to the value of quantitative cytological study of the adenohypophysis for the estimation of thyroid function. Previous work by Hohlweg and Junkmann (1933) and Lebedewa (1936) pointed to the fact that complete absence of thyroid tissue leads to complete degranulation of the acidophils of the rat anterior pituitary. In a series of studies, Griesbach (1943, 1945, 1946) correlated the percentage of acidophils with the level of circulating thyroid hormone and demonstrated that only in the case of total thyroidectomy was degranulation of the acidophils complete. This work has been confirmed in this laboratory (unpublished observations) and has now been observed in those rats which were *radiothyroidectomized.* In those rats which were found to have no thyroid

tissue upon complete serial section of the neck region and which had, in addition, the characteristics of new-born thyroidectomies as described by Salmon (1936) and Scow and Simpson (1945), among others, the acidophil count in the pituitary was found to be in the region of one per cent or less. In those rats which were found to contain only a few surviving follicles, however, the acidophils were present in from 15–40 per cent of the total cell count. Although the presence of the acidophils in the degranulated condition can be demonstrated by special techniques (Severinghaus, 1932), or by regranulation experiments with thyroxine (Griesbach, 1946), the usual practice of including degranulated acidophils in the total chromophobe count has been followed in the present study.

Aside from its simplicity, radiothyroidectomy has certain advantages over the surgical procedure for excision of the gland. These are: low mortality (one of the 28 I^{131}-treated rats died); no adverse bone effects due to refrigeration anesthesia (Scow and Simpson, 1945; Scow, 1949); and the relative ease of maintenance of the treated animals. An interesting feature of this procedure is the survival of the parathyroids even when the dose of I^{131} injected is large enough to wipe out all thyroid tissue completely.

ACKNOWLEDGMENT

We are indebted to Mr. V. Aman for the preparation of the roentgenograms used in this study.

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SPECIFICITY OF THE INTRAUTERINE TEST FOR PROGESTERONE[1]

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IN AN EARLIER paper (Hooker and Forbes, 1947) a bioassay that detects 0.0002 μg of progesterone was described. The sensitivity of the test readily permits the measurement of levels of this hormone in blood (Hooker and Forbes, 1947, 1949) and other body fluids, and offers promise of being useful in investigating several aspects of the metabolism of progesterone. To be of more than seriously limited value, however, an assay must be more or less specific, irrespective of its sensitivity. It has already been reported (Hooker and Forbes, 1947) that estrone, α-estradiol, desoxycorticosterone acetate, and testosterone failed to elicit the reaction used as a test for progesterone. The specificity of the test has been examined further by administering several additional substances.

All of the substances tested are listed in Table 1. Included are (1) compounds reported to duplicate the action of progesterone in other tests, (2) compounds considered to be metabolites or possible precursors of progesterone, (3) compounds closely related to progesterone chemically, and (4) compounds that might be expected to accompany progesterone in biological material.

The assay involves injection of a solution of the material to be tested directly into a segment of the uterus of an ovariectomized mouse. The response to progesterone consists of transformation of some of the endometrial stromal nuclei, which in the castrate are

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Progesterone, desoxycorticosterone acetate, dehydroisoandrosterone, anhydrohydroxyprogesterone, Δ^5-pregnenolone, pregnanedione, allopregnanedione, allopregnanolone, estradiol dipropionate, and estradiol benzoate were furnished by Dr. Erwin Schwenk of the Schering Corporation; desoxycorticosterone, by Dr. Albert Segaloff; α-estradiol, androsterone, testosterone, and testosterone propionate, by the late Mr. Robert A. Mautner, and methyl testosterone, cis-testosterone, testosterone dipropionate, by Dr. Ernst Oppenheimer, of Ciba Pharmaceutical Products, Inc.; pregnanediol, by Dr. A. Stanley Cook of Ayerst, McKenna and Harrison, Ltd.

The adrenal extracts were obtained by Dr. Abraham White from The Wilson Laboratories. The oily extract was listed as containing 40 rat units (Survival-Growth) in each ml., and 1 ml. of the undiluted aqueous extract was listed as having been derived from 75 gms. of suprarenal tissue.

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shrunken, dense, and fusiform, into plump, slightly elongated oval nuclei with conspicuous nucleoli and fine, evenly dispersed chromatin particles. The details of the method have been described before (Hooker and Forbes, 1947), and need not be repeated here. With three exceptions all of the substances tested were dissolved in sesame oil. Water was the solvent for the ascorbic acid and the aqueous adrenal extract, and an unidentified oil was the solvent for the oily

TABLE 1. SUCCESS (+) OR FAILURE (−) OF COMPOUNDS IN EVOKING A NUCLEAR RESPONSE IDENTICAL WITH THAT PRODUCED BY PROGESTERONE

	mg./I.U. (Selye & Masson)	Intrauterine injection, micrograms		Subcutaneous administration, milligrams	
		−	+	−	+
Progesterone	1	.0001	.0002	0.375, 0.875, 0.875	1.5, 0.75 0.375, 0.375
Anhydrohydroxyprogest. (Ethinyl testosterone)	10	Less than .006		Small amt. from pellets (2 mice)	
Desoxycorticosterone acetate	10	6, 3, 0.6		5, 2.5, 1.25	5, 2.5
Desoxycorticosterone		6, 3, 1.5			
Methyl testosterone	30	15, 3, 0.6		25, 12.5, 6.25, 1,	
Testosterone prop.	50	15, 15, 3, 3, 0.6, 0.6		9.5, 3.125, 1.563, 1, 0.75,	25, 12.5, 6.25, 6.25
Testosterone diprop.		3, 0.6, 0.12			
Δ^5-pregnenolone	40–100	5.7, 2.9, 1.4		5, 5, 2.5	
Testosterone	140–200	0.48, 0.24, 0.16, 0.12		2.5, 2.5, 2.5	
Cis-testosterone		Approx. 8, 8, 4, 4			
Pregnanediol	0	Unknown concn.			
Pregnanedione	0	3, 1, .005	12?, 6?		
Allopregnanedione		6, 3, 1, .005	12?		
Allopregnanolone		12, 6, 3, 1, 0.005			
Androsterone	0	6, 6, 3, 1.5			
Dehydroisoandrosterone	0	6.3			
Cholesterol	0	5.2, 2.6, 1.3			
Oily adrenal cortical ext.		Undild., ½ dild.			
Aqueous adrenal cortical ext.		½ dild.	Undild.?		
Estrone		0.6, 0.3, 0.15			
Estradiol	0	.000000023–.00075 (36 mice)			
Estradiol benzoate				.0488, .0488, .0488	
Estradiol dipropionate				.0001 (6 mice)	
Ascorbic acid in water		171, 85.5			

adrenal extract. In each instance the volume of vehicle injected was 0.0006 ml. The quantities in micrograms of each substance given are shown in the table.

As indicated in the table, progesterone was the only one of the 24 substances that provoked changes in the endometrial stromal nuclei that clearly satisfied all of the criteria of the assay. Many of the other substances provoked changes of various kinds in the stromal nuclei, most of which were quite distinct from the changes elicited by progesterone. Pregnanedione in amounts of 12 and 6 μg, allopregnanedione in a dose of 12 but not 6 μg, and the undiluted aqueous adrenal extract evoked changes that were sufficiently similar to those of progesterone that the reactions had to be recorded as questionable positives; these responses, however, could not be read as clear-cut positive reactions.

Inasmuch as quantities of the order of 30,000 to 60,000 times the

effective dose of progesterone gave but a questionably positive reaction, there can be some doubt that pregnanedione and allopregnanedione duplicated the action of progesterone in the test. The questionably positive effect of the undiluted aqueous adrenal extract could possibly have been due to a small quantity of progesterone in the extract. Beall (1938) and Von Euw and Reichstein (1941) have isolated progesterone in adrenal extracts, and a computation suggests that the assay here would be positive if the extract tested contained but 1 mgm. of progesterone for each 225 kg. of adrenal tissue processed. It is possible, however, that one of the "true" cortical steroids almost duplicated the effect of progesterone in the test.

Whether the test is completely specific for progesterone can probably never be ascertained rigorously; an almost endless series of compounds in a variety of doses would have to be examined. The group of substances tested, however, include most of the substances that offer a possibility of being a source of error, and only progesterone gave an unequivocally positive reaction.

In addition to intrauterine injection, certain of the substances were also given systemically, as shown in the table. The anhydrohydroxyprogesterone was given in the form of a subcutaneous pellet (one mouse) and an intrasplenic pellet (one mouse), and the animals were killed for study after 14 days. The other compounds given systemically were dissolved in sesame oil and injected subcutaneously in five or six daily doses. The total quantity administered is listed in the table.

Testosterone propionate in amounts of 6.25 mgm. or higher regularly and desoxycorticosterone acetate in amounts of 2.5 mgm. and higher sometimes duplicated the action of progesterone on the stromal nuclei (Hooker, 1945). The other compounds in the quantities administered failed to reproduce the action of progesterone. Neither desoxycorticosterone acetate nor testosterone proprionate gave a positive test when administered topically, and neither was effective when given systemically until 16.6 and 6.6 times the minimal effective amount of progesterone was administered.

These observations seem to be more consistent with the possibility of conversion of a fraction of the subcutaneously injected compound into progesterone than with progestational activity being an inherent property of these two compounds as such. It would be interesting to know whether the several compounds found by Selye and Masson (1943) to exhibit luteoid activity when given systemically to rabbits are also effective when applied directly to the endometrium of the rabbit. The multiple and overlapping actions of many steroids as studied by many investigators could conceivably be the result of metabolic interconversion (large quantities are almost always necessary) rather than inherent pharmacologic properties of the various compounds.

Questions of this sort are important in determining what chemical configuration is basic to any particular action of a steroid hormone. The limited data presented here perhaps suggest that the molecular morphology of progesterone is necessary in this test, and that modifications, however slight, abolish the action studied.

SUMMARY

The specificity of a sensitive bioassay for progesterone that depends upon intrauterine injection in mice has been examined by administering 24 substances. Included were compounds that are progestational by other tests, compounds that may be metabolites or precursors of progesterone, and compounds that might be expected to accompany progesterone in biological materials. Only progesterone gave a clearly positive reaction. Pregnanedione and allopregnanedione in large doses and an undiluted aqueous adrenal extract provoked questionable positive responses.

Certain of the compounds were also given systemically. Desoxycorticosterone acetate and testosterone propionate in large doses duplicated the action of progesterone upon the endometrium.

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THE EFFECT OF VARYING LEVELS OF THYROIDAL STIMULATION ON THE ASCORBIC ACID CONTENT OF THE ADRENAL CORTEX

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E. LANSING, MICH.

THERE IS considerable evidence suggesting an interrelationship between the thyroid gland and the adrenal cortex. Hoskins (1910), Herring (1917), Cohen (1935), Schmidt and Schmidt (1938), Deane and Greep (1947), and others have reported that following the administration of thyroidal substances to animals, the adrenals enlarge, the hypertrophy being restricted to the cortex.

Leblond and Hoff (1944) noted a decrease in the size of the adrenals in rats receiving goitrogenic sulfa drugs or thiouracil. This was confirmed by Baumann and Marine (1945) when they noted involution of the adrenals to half their former size in rats fed thiouracil for four months.

From these reports it seems apparent that the functional activity of the adrenal cortex is interrelated in some manner with the thyroid secretion level. Very little information has been reported however, that specifically relates the adrenal cortical secretion to the thyroid status.

It has been demonstrated recently by Long (1947) and Sayers and Sayers (1948) that the adrenal cholesterol and ascorbic acid concentrations provide a reliable index of the functional activity of the adrenal cortex. In the work to be reported, the ascorbic acid content of the adrenals was used to determine the influence of varying degrees of thyroidal stimulation on adrenocortical function in the male white rat.

EXPERIMENTAL

Except as otherwise stated, male rats of the Michigan State College strain were used. After a given experimental treatment, the animals were killed by decapitation, and the adrenals were dissected out, trimmed cleanly, and weighed to the nearest tenth of a milligram. Following this, they were placed in 50 cc. centrifuge tubes containing about 3.5 gm. of coarse sand saturated with 1 cc. of 3% metaphosphoric acid that was prepared by the method of Briggs (1938). The

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glands were macerated by gentle grinding with a glass rod. The resulting mass was washed with two 10 cc., and one 5 cc. portion of 3% metaphosphoric acid, and centrifuged after each addition of acid. The resulting supernatant liquid was freed of turbidity by mixing with celite and subsequent centrifigation.

A twenty cc. aliquot of the clear extract was then adjusted to a pH of 3.0–3.5 with 10 cc. of a sodium citrate buffer. The ascorbic acid content of the buffered extract was determined by the method of Mindlin and Butler (1937) as modified for plant and animal tissues by Bessey (1938).

The Response of the Adrenals to a Constant Level of Thyroidal Stimulation as a Function of Time

It has been demonstrated by Long (1947) and Sayers and Sayers (1948) that time relationships are very important in the response of

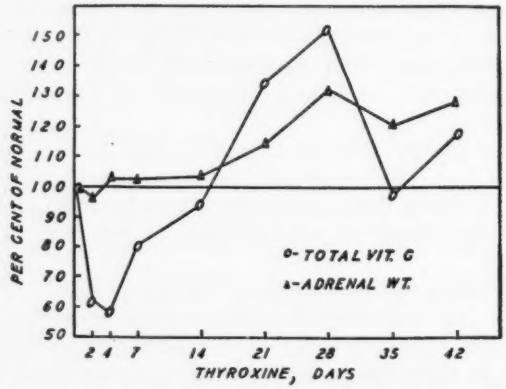

FIG. 1. Changes in the weight and ascorbic acid content of the adrenals brought about by a daily dosage of 20 μg. of thyroxine per 100 gm. body weight during a six-week period.

the adrenals to acute stress. This experiment was designed to investigate the response of the adrenals to the more prolonged stimulation of thyroxine injections.

Eight groups of at least 5 rats each were injected daily with a solution of d, 1-thyroxine at a dosage of 20 ug. per 100 gm. body weight, this dosage being approximately 5 times the estimated thyroid secretion rate of this strain of rats (Reineke, unpublished data).

Four groups of controls were used. Group 1 was killed at the start of the experiment, and the second, third, and fourth groups, were killed after 3, 4, and 6 weeks respectively. The last three groups received daily injections of 0.1 cc. of physiological saline solution per 100 gm. body weight.

Although there was some variation between the different control groups, no consistent trends in either adrenal weight or ascorbic acid were noted. Consequently the values for adrenal weight and ascorbic acid, respectively, were pooled and are shown graphically in figure 1 as the base line or 100%. The mean control adrenal weight with standard deviation was 42.0 ± 5.98 mg. The value for ascorbic acid was 194.8 ± 52.7 ug. All the values in the experimental groups were calculated as percentages of this base.

Values obtained 2 and 4 days after thyroxine injections were begun showed a pronounced decline in the ascorbic acid content of the adrenals (Fig. 1). This was followed by a return to normal levels and finally by considerable overcompensation, as shown by the pronounced rise above normal after 28 days of stimulation. Coincident with the leveling off of the adrenal weight at a point considerably above normal, the ascorbic acid content showed a trend toward normal values.

Effect of Varying Levels of Thyroidal Stimulation on Adrenal Weight and Ascorbic Acid Content

This experiment was designed to determine the effects of varying levels of thyroidal stimulation on the response of the adrenals at 4 weeks.

Koger and Turner (1943) showed that dosages of thyroprotein in the feed, ranging from 0.01% to 0.16%, produced noticeable and largely adverse effects in male rats with regard to growth, the higher levels showing a definite toxic effect. The effects on the adrenals varied with two strains of rats employed. One strain showed hypertrophy of the adrenals which "paralleled roughly the level of thyroprotein fed." Females of the other strain showed this hypertrophy but, surprisingly, the males did not show an increase in adrenal weight, and with some of the dosages used, the adrenals showed a statistically significant decrease in size.

In this experiment a thyroactive iodinated protein "Protamone"[2] was fed in dosages corresponding to the first five levels used by Koger and Turner (1943) which ranged from 0.01% to 0.16% mixed in the feed and given *ad libitum*. After 4 weeks of treatment (Table 1, Part 1) there was no significant trend in either the adrenal weight or ascorbic acid content of any of the experimental groups when compared with the controls. This would indicate that the dosage range was too low to evoke a response from the adrenals in this strain of rats.

In order to determine the effect of higher levels of thyroidal stimulation, 5 groups of rats were placed on daily injections of thyroxine ranging from 5 ug. to 80 ug. per 100 gm. body weight for a period of 28 days. Five ug. of thyroxine per 100 gm. body weight had no significant effect on either adrenal weight or vitamin C content after

[2] Kindly supplied by the Cerophyl Laboratories, Kansas City, Missouri.

four weeks of treatment. At dosages of 10 and 20 ug. per 100 gm. body weight, there was a progressive increase in the size and ascorbic content of the adrenals. Forty ug. of thyroxine per 100 gm. body weight caused no further increase in either adrenal weight or ascorbic acid content, when compared with the group receiving 20 ug. daily. When the dosage level of thyroxine was increased to 80 ug. per 100 gm. body weight, however, there was an increase of 52% in weight, and 97% in ascorbic acid content of the adrenals of the hyperthyroid rats when compared with the controls.

It has been reported by numerous authors that the adrenals of male animals are smaller than those of the female. To explore the possibility of sex differences in vitamin C in this experiment, a group of 8 females of approximately the same age, were killed concurrently with the normal controls (Table 1, part 2). It was found that the female adrenals were significantly heavier than the males. The ascorbic acid content was also slightly higher than that of the males, but this difference was not statistically significant.

Determination of the Increase in the Secretion Rate of the Adrenal Cortex Due to Hyperthyroidism

There is extensive literature reporting that the administration of adrenocortical extracts, and hormones structurally related to those secreted by the adrenal cortex, will produce adrenocortical atrophy. (Selye, 1940; Carnes, *et al.* 1941; Ingle, 1939). Ingle *et al.* (1937) postulated that when there is an amount of cortin present in the body above physiological requirements, the output of adrenocorticotrophic hormone is suppressed. Ingle (1938) further observed that when the hypophysis is absent, and the size of the adrenals is maintained with adrenocorticotrophic hormone, cortin has no effect on the adrenals.

From these facts a method for estimating the increase in the adrenocortical secretion rate in hyperthyroid rats may be suggested. As has been shown in the preceding experiment, the maximum response of the adrenals to a dose of 20 ug. of thyroxine per 100 gm. body weight per day, is seen at the fourth week. It was thought that if this response could be prevented, wholly or in part, by the simultaneous administration of adrenocrotical hormone, the dose of hormone necessary to accomplish this would be an indication of the increase in the secretion rate of the adrenals caused by this degree of thyroidal stimulation. In addition this would be prima-facie evidence that the adrenals are in a state of hypersecretion in a hyperthyroid animal. The hypersecretion is regarded as evidence for an increased requirement for the adrenocortical hormone in these animals. Evidently more adrenocorticotrophin is secreted when thyroxine is administered, since the adrenals are enlarged.

TABLE 1. EFFECT OF VARYING LEVELS OF THYROIDAL STIMULATION ON ADRENAL SIZE AND ASCORBIC ACID CONTENT DURING A 28 DAY PERIOD

Group	No. of animals	Body wt. gm.	Adrenal wt. mg.	Total vit. C. ug.	% Protamone in feed
Part 1. Effect of Feeding Protamone at Physiological Levels of Stimulation					
1	9	238±24.0	38.5± 5.3	180.4±22.6	0.01%
2	8	231±22.0	35.0±11.8	142.3±27.8	0.02%
3	9	231±36.0	41.3± 4.4	186.7±26.4	0.04%
4	10	237± 7.0	43.7± 2.2	208.9±40.7	0.08%
5	10	219±23.0	40.3± 7.5	185.9±46.2	0.16%
Controls	9	243±26.0	39.9± 5.2	170.5±27.8	No treatment
Part 2. Effect of Injections of Thyroxine at High Dosage Levels (ug. Thyroxine/100 gm. Body Wt.)					
1	4	257±12.0	42.3± 3.3	204.6±26.4	5
2	4	251±26.0	49.8± 3.3	246.5±16.0	10
3	5	271±19.0	55.5± 6.3	296.0±15.4	20
4	5	236± 9.0	55.3± 5.5	282.4±38.5	40
5	5	223±19.0	63.8± 5.9	401.7±31.3	80
Pooled Controls	22	260±20.0	42.0± 5.9	194.8±52.7	0.1 cc. Physiological Saline/100 gm. Rat
Normal Females	8	220±19.0	52.4± 6.8	229.9±22.4	No treatment

TABLE 2. THE INHIBITORY EFFECT OF ADRENOCORTICAL HORMONE ON THE ADRENOCORTICAL HYPERTROPHY ELICITED BY THYROXINE STIMULATION

Group*	Body wt. gm.	Adrenal wt. mg.	Total vit. content ug.	Treatment**—units of Eschatin/day
1	276±12.7	52.8±5.9	289.6±30.1	10
2	262±11.7	46.9±4.5	249.2±11.7	20
3	261± 9.6	46.8±5.7	261.2± 9.6	40
4	271±19.0	55.5±6.3	296.0±15.4	No Eschatin.

* Five rats were included in each group.
** Twenty ug. thyroxine was given daily per 100 gm. body weight for 28 days. During the last 8 days of this period Eschatin was given concurrently with the thyroxine in the amounts specified.

Accordingly, the adrenocortical extract "Eschatin"[3] was injected into three groups of five rats each, which had received injections of 20 ug. of thyroxine per 100 gm. body weight daily for 20 days. Group 1 received 10 units of Eschatin daily. Groups 2 and 3 received 20 and 40 units per day, respectively. The animals were kept on simultaneous injections of thyroxine and cortical extracts for 8 days, after which they were sacrificed, and the adrenals were removed and analyzed for ascorbic acid.

Group 1 which received 10 units of cortical extract per day showed

[3] We are indebted to Dr. H. O. Von Rosenberg, Parke, Davis and Company, Detroit, Michigan, for supplying the cortical extract, "Eschatin," used in these experiments.

no significant decrease in either adrenal weight or ascorbic acid content, when compared with the group receiving the same amount of thyroxine alone for 28 days.

The two groups receiving 20 and 40 units of cortical extract per day, respectively, in addition to thyroxine injections, showed no significant differences when compared with each other in either adrenal weight or ascorbic acid. When these two groups were compared with the group receiving thyroxine alone for 28 days, a highly significant decrease both in adrenal size and ascorbic acid was observed.

It seems evident from these data, that the increase in the secretion rate of adrenocortical hormones in rats receiving 20 ug. of thyroxine per 100 gm. body weight per day, is between 10 and 20 dog units per day. Above the dosage of 20 units per day, additional adrenocortical hormones did not show a perceptible effect under our experimental conditions.

DISCUSSION

It has been demonstrated in the experimental work of this report that time relationships are very important in the response of the adrenals to prolonged stimulation with thyroxine. Assuming that the vitamin C content of the adrenals is an index of secretion, it would appear that the adrenals are discharging increased amounts of cortical hormones into the blood stream in the earlier stages of induced hyperthyroidism. At this stage the adrenal cortex has only a normal amount of secretory tissue present. Consequently, the discharge of hormone exceeds its rate of synthesis in the gland as evidenced by the pronounced decline in ascorbic acid content after 2 and 4 days of stimulation. Concurrently with the development of more secretory units in the adrenal at the third week, as shown by increased weight, there is an increase in the vitamin C content above normal values. This means that not only is there an increased rate of secretion, but also an increased rate of hormone synthesis.

At four weeks there is an oversupply of adrenocortical hormones, and when these are discharged, they inhibit further secretion of adrenocorticotrophic hormone by the anterior pituitary. This is followed by a decline in adrenal vitamin C and a noticeable decrease in adrenal weight. At the sixth week, a balance may be presumed to be established, in which the size of the adrenals and the rate of hormone synthesis and secretion is adequate for the degree of thyroidal stimulation to which the animal is being subjected. However, the mechanism whereby this balance is brought about remains to be elucidated.

Evidence is advanced to show that the level of thyroidal stimulation determines to some extent, the size and secretory activity of the adrenal cortex. There is a rather narrow dosage range in which thyroidal stimulation has no demonstrable effect on the adrenals. This range may be considered to be the "Physiological Range," or the dosage approximating the animal's own thyroid secretion rate.

When measuring the hormonal activity of the adrenal by ascorbic

acid assay, it might be questioned whether an increased amount of vitamin C in the adrenal means increased synthesis and subsequent liberation of adrenocortical hormones, or whether the increase in ascorbic acid content is merely indicative of increased storage. In favor of the first interpretation is the fact that the initial response of the adrenal to adequate thyroidal stimulation is a decrease in ascorbic acid. Only when enough secretory units are laid down so that the adrenals can balance the demand for cortical hormones does the ascorbic acid content show an increase.

Strong evidence that the adrenals of a hyperthyroid rat are in a state of hypersecretion is provided by the fact that 10–20 dog units of cortical hormone are required daily to bring the pituitary-adrenal mechanism back into normal balance.

SUMMARY

The effect of thyroidal stimulation on the adrenal ascorbic acid content and adrenal weight of the male white rat has been studied. The ascorbic acid content of the adrenal decreases to minimal values after 4 days of thyroxine administration. This is followed by progressive increases, both in adrenal weight, and ascorbic acid content which reach a maximum at 4 weeks. Following this, there is a general levelling off at the fifth and sixth weeks. The response of the adrenals to different adequate dosage levels of thyroxine is one of a progressive increase in size and ascorbic acid content at 4 weeks. The increased secretion rate of the adrenals in a hyperthyroid rat has been estimated to be equivalent to the amount falling between 10 and 20 dog units of adrenocortical hormones.

ACKNOWLEDGMENT

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COMPARATIVE ORAL THYROXINE-LIKE ACTIVITY OF NATURAL AND SYNTHETIC THYROPROTEINS AND OF DL-THYROXINE STUDIED WITH THE GOITER PREVENTION METHOD[1]

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LOS ANGELES

INTRODUCTION

In a previous report (Frieden and Winzler, 1948) it was concluded that parenterally administered natural thyroid proteins exert greater thyroxine-like activity than could be attributed to their L-thyroxine content. Synthetic thyroproteins, on the other hand, showed only one-half the parenteral activity expected from their L-thyroxine analysis. The convenience, precision (see Hutchson, 1948) and sensitivity of the goiter prevention method in the physiological range of thyroxine dosage suggested its application to the determination of the relative oral thyroxine-like activity of natural and synthetic thyroproteins and of DL-thyroxine. This is a factor of some importance in research and in thyroid therapy.

METHODS AND MATERIALS

The procedure for the biological assay for thyroxine-like activity was essentially the same as that described earlier. The thyroid glands were weighed on removal from the test animal after 15 days on a stock diet containing 0.3–0.5% thiouracil incorporated into a stock diet with a daily oral administration of the test substance. The biological activity of the preparation was estimated to the nearest 0.05 γ from a plot of the thyroid weight against parenteral DL-thyroxine controls for each individual experiment. In these experiments male rats weighing 100–200 gm. were used, and all test materials were orally administered except for certain intraperitoneally injected DL-thyroxine controls.

The thyroactive substances used in this study were the various natural thyroid preparations and the synthetic thyroproteins described previously byl Frieden and Winzler (1948). The test materials were dissolved or suspended

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in an aqueous solution containing 10% glucose, 0.50% chlorobutanol and 0.10% $NaHCO_3$. DL-thyroxine was dissolved in a minimum amount of 0.1 N NaOH, the pH adjusted to 8.0 ± 0.5 and the solution made up to volume with the above glucose solution in the case of the solutions used for oral administration or with distilled water for those solutions used for intraperitoneal injection. Volumes of approximately 0.10 ml. per 100 gm. body weight of these mixtures could be satisfactorily administered once daily by mouth through a 0.25 ml. syringe fitted with a polished blunt 20 gauge needle.

RESULTS AND DISCUSSION

The results obtained in this survey of the oral activity of various thyroactive materials are summarized in Table 1. Sample data from one of six independent experiments is shown in Figure 1. These data show that under these conditions, orally administered thyroxine is about one-half as active as when given parenterally. It is emphasized that the doses of thyroxine employed in these experiments were in

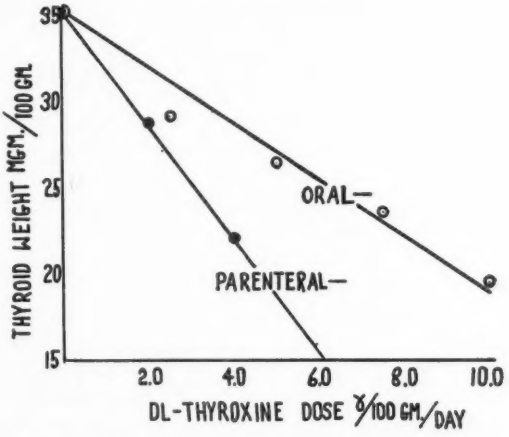

FIG. 1. Relative activity of orally and parenterally administered DL-thyroxine as indicated by a plot of DL-thyroxine dose against thyroid weight. Groups of six 100–200 gm. male rats were maintained on a 0.3–0.5% thiouracil diet for 15 days during which time they were given daily doses of DL-thyroxine by the indicated route.

the physiological range and are not directly comparable to most previous work[2] in which much greater doses were administered to produce significant effects on oxygen consumption and other less sensitive methods for measuring thyroxine-like activity. One factor contributing to the high oral activity of DL-thyroxine in these experiments is the fact that the substance was in solution when given. While the thyroxine was probably precipitated at the pH of the stomach,

[2] Previous work regarding the relative oral activity of thyroactive materials is summarized in a number of texts including those of Kendall (1929), Harington (1933), Means (1937), Elmer (1938), and Salter (1940).

TABLE 1. SUMMARY OF EXPERIMENTS ON THE THYROXINE-LIKE ACTIVITY OF NATURAL AND SYNTHETIC THYROPROTEINS WHEN ADMINISTERED ORALLY

0.3%–0.5% thiouracil diet plus	Per cent total iodine	Per cent thyroxine	No. of groups tested	Total no. of animals tested	Range of dosage: γ-DL-thyroxine or equivalent per 100 gm. wt.	Biological Response[1]: γ-thyroxine in dose	Per cent of parenteral activity[2]
DL-thyroxine	65.4	100.0	9	54	2.5 –10.0	0.50 ±0.029[2]	49
Desiccated thyroid—1	0.52	0.26	6	35	0.75– 2.0	2.85 ±0.160	71
Desiccated thyroid—2	0.64	0.31	4	25	0.75– 1.0	2.65 ±0.24	72
Product A	0.99	0.46	3	15	1.00– 2.0	3.00 ±0.105	98
Product B	0.27	0.13	3	19	1.00– 2.0	3.10 ±0.110	71
Synthetic thyroprotein —1	10.7	2.6	4	22	12.0 –30.0	0.143±0.0012	13.6
Synthetic thyroprotein —2	8.6	2.9	1	6	20.0	0.120±0.018	14.1

[1] Estimated to the nearest 0.05γ from parenteral DL-thyroxine dose-response curve for particular experiments.

[2] Standard error of the mean.

[3] Calculated from the expression: $\frac{\text{oral biological response}}{\gamma\text{-thyroxine in dose}} \times 100 / \frac{\text{parenteral biological response}}{\gamma\text{-thyroxine in dose}}$ the latter ratio from a previous publication (Frieden and Winzler, 1948).

the flocculent nature of such a precipitate would have facilitated resolution in the duodenum.

Table 1, column 6, lists the ratio of the biological activity of the indicated thyroprotein to the chemical thyroxine content of the dose. If it be assumed that L-thyroxine possesses twice the biological activity of DL-thyroxine, this ratio should have a maximum theoretical value of 2.0. The results obtained with natural thyroid proteins indicate that even when given orally, these materials exhibit ratios significantly greater than 2.0, averaging about 2.9.[3] Thus desiccated thyroids 1 and 2 and commercial product B gave ratios of 2.85 ,2.65 and 3.10 respectively which represented 71%, 72%, and 71% of their parenteral ratios as indicated in column 7. Commercial product A, with the highest thyroxine content and greatest solubility of any of the natural thyroid proteins, retained 98% of its parenteral activity. Even larger ratios of 5.8 result from calculating the ratio of the biological activity in terms of *oral* DL-thyroxine activity to the chemical thyroxine content. Since it is reasonable to assume that thyroglobulin cannot be absorbed intact, the above data suggests a high order of digestibility of natural thyroid proteins.

Synthetic thyroproteins, in contrast, were considerably less potent orally than parenterally. Two preparations showed only about one-seventh of their parenteral potency in agreement with the results obtained by Turner and Reineke (1946) on the utilization of synthetic thyroprotein by sheep. Explanations for this poor utilization of synthetic thyroproteins might be found in the large steric hindrance to enzyme action resulting from the high percentage of iodine or in the

[3] Explanations for the fact that natural thyroid proteins consistently show more thyroxine-like activity than indicated by their L-thyroxine content have been summarized recently by Frieden and Winzler (1948) and by Hamilton, Albert, and Power (1948) in addition to earlier discussion in the texts mentioned in footnote 2.

elimination of the sensitivity of many peptide linkages to the cleaving action of the endopeptidases, chymotrypsin and pepsin, which require intact tyrosine units. Other gross structural changes possibly attributable to iodine cannot account for our observations because of the insensitivity of casein to denaturation and the fact that substrate denaturation frequently increases ease of proteolysis.

SUMMARY

The comparative oral thyroxine-like activity of physiological range doses of DL-thyroxine and natural and synthetic thyroproteins has been measured using the goiter prevention method. The following conclusions were drawn:

DL-thyroxine demonstrated half of its parenteral activity when administered orally as the soluble sodium salt.

Four different natural thyroid preparations assayed 70%, 71%, 72%, and 98% of their parenteral potency. This activity was greater than could be attributed to the L-thyroxine content of the test substances when compared with parenterally administered thyroxine.

Synthetic thyroproteins showed only one-seventh of their parenteral activity, indicating poor absorption from the small intestine.

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OCCURRENCE AND DETERMINATION OF CONJUGATED SULFATES OF ESTROGENS IN URINE FROM PREGNANT WOMEN

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THE ISOLATION of estriol glucuronide from pregnant women's urine (Cohen, Marrian, and Odell, 1936) and estrone sulfate from pregnant mares' urine (Schachter and Marrian, 1938) demonstrated the fact that the estrogens and their metabolites may be excreted as water-soluble esters of glucuronic or sulfuric acid. Estrogens, other than estriol, have been found in human pregnancy urine, but their conjugates have never been isolated. Cohen and Marrian (1936) recognized the importance of this problem and Marrian (1937) wondered whether the estrone in human urine will be found to be conjugated with glucuronic acid like estriol or with sulfuric acid, as it is in pregnant mares' urine. Butenandt and Hofstetter (1939), although never actually isolating estrone sulfate from human pregnancy urine, concluded on the basis of solvent fractionations as well as chromatographic studies, that estrone was excreted by the human as the sulfuric acid ester because the partitioning properties of the estrone fraction resembled those of synthetic estrone sulfate. Crepy (1946) incubated estrone, estradiol and estriol with liver slices and concluded that estradiol and estriol were conjugated for the most part, with glucuronic acid, whereas, estrone seemed to be conjugated with another substance resembling sulfuric acid.

The results of Peterson et al. (1938) and Venning et al. (1942) showed that the androgenic steroid hormones in human male urine may be conjugated with either glucuronic acid or sulfuric acid. Wilder Smith (1948) claimed indirect evidence for the excretion of synthetic estrogens conjugated with sulfuric as well as glucuronic acid.

The present paper concerns itself with studies to determine the proportion of estrogenic conjugates that occur as the sulfate ester in pregnant women's urine. Utilizing the specific phenolsulfatase present in extracts of aspergillus oryzae (Abbot, 1947; Cohen and Bates, 1949), the estrogens conjugated with sulfuric acid were hydrolyzed by Mylase P and became extractable from water with ether or toluene, whereas, the estrogens conjugated with glucuronic acid were

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not hydrolyzed by the enzyme and remained soluble in water. It was found that both the weak phenolic and the strong phenolic estrogens are excreted, in part, as conjugated sulfates.

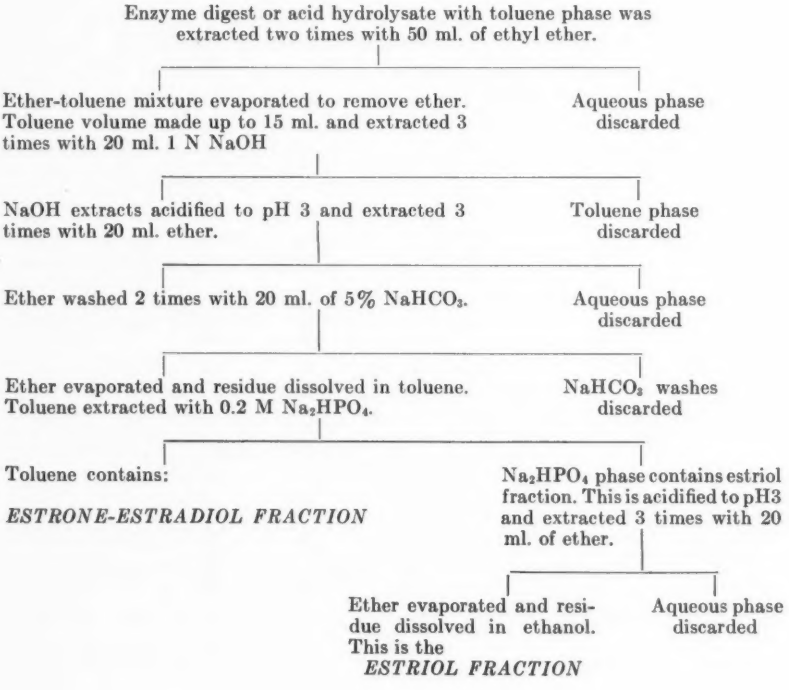
MATERIALS AND METHODS

Urine from women who were in the 7th–9th month of pregnancy was used. When urine was used as substrate, the urine was collected under toluene and the toluene layer withdrawn to remove the free estrogens before digestion with Mylase P. In the cases where butanol extracts were used, the urine was collected under butanol, and then extracted with butanol. After the butanol was removed in vacuo, the residue was dissolved in water (pH 6–7) and the free estrogens were removed by extraction with ether, leaving a clear aqueous fraction which was used as substrate for enzyme and acid hydrolysis as well as for control tests.

Mylase P[1] was used as a source of phenolsulfatase. Enzyme digestions performed under a layer of toluene at 50°C. and pH 6.0 for a period of 24 hours, using 200 mg. of Mylase P per 100 ml. of urine or its equivalent. These conditions have been found to result in complete hydrolysis of estrogenic sulfates (Cohen and Bates, 1949). Controls for the enzyme digestion were prepared and processed exactly as was the enzyme digest except that no

FIG. 1

Procedure for Extraction of Estrogens


Enzyme digest or acid hydrolysate with toluene phase was extracted two times with 50 ml. of ethyl ether.
Ether-toluene mixture evaporated to remove ether. Toluene volume made up to 15 ml. and extracted 3 times with 20 ml. 1 N NaOH
Aqueous phase discarded
NaOH extracts acidified to pH 3 and extracted 3 times with 20 ml. ether.
Toluene phase discarded
Ether washed 2 times with 20 ml. of 5% NaHCO3.
Aqueous phase discarded
Ether evaporated and residue dissolved in toluene. Toluene extracted with 0.2 M Na2HPO4.
NaHCO3 washes discarded
Toluene contains:
ESTRONE-ESTRADIOL FRACTION
Na2HPO4 phase contains estriol fraction. This is acidified to pH3 and extracted 3 times with 20 ml. of ether.
Ether evaporated and residue dissolved in ethanol. This is the
ESTRIOL FRACTION
Aqueous phase discarded

enzyme was added. Blank tubes containing enzyme alone gave negative tests for estrogens.

Acid hydrolysis of urine was performed, after the addition of 15 volumes per cent hydrochloric acid, C. P., by boiling for fifteen minutes under a layer of toluene. Acid hydrolysis of the butanol extract preparations was performed by acidifying the solutions to pH 0.8 and boiling them for fifteen minutes under a layer of toluene.

Extraction of enzyme digests and acid hydrolysates were carried out according to the procedure in Figure 1. The phenolic fraction was separated into an estriol fraction and an estrone-estradiol fraction, using 0.2 M. disodiumphosphate, as recommended by Friedgood et al. (1948). In one case (Table 1, D.) the fractions were separated using the chromatographic method of Stimmel (1946)

Estrogen determinations were made by the fluorimetric method of Bates and Cohen (1947). An estrone standard was used for the estrone-estradiol fraction and an estriol standard for the estriol fraction. In the case of the estriol fraction, confirmatory positive tests by a modified Kober method (Cohen and Bates, 1947) and negative tests by a modified Zimmerman method (Holtorff and Koch, 1940) were obtained. Because of the low estrogen content of the estrone-estradiol fraction, Kober and Zimmerman determinations were not made.

RESULTS

The data are shown in Table 1. The percentage of estrogen conjugated with sulfuric acid was calculated using the acid hydrolysis value as the value for the total estrogen content of each fraction and the difference between the enzyme and control values as the amount hydrolyzed by the enzyme. It can be seen that the amount released

TABLE 1. PERCENTAGE OF ESTROGENS CONJUGATED AS THE SULFATE IN HUMAN URINE OF LATE PREGNANCY AS ESTIMATED BY HYDROLYSIS WITH MYLASE P. THE FREE ESTROGENS WERE REMOVED PRIOR TO TREATMENT BELOW.

Patient	Pregnancy stage	Nature of substrate	Urine vol. equivalent	Method of hydrolysis	Estrone-estradiol as estrone	Estriol as estriol	Estrone-estradiol fraction	Estriol fraction
	months				micrograms	micrograms		
A	8	Urine	100	Enzyme	70	400	46%	20%
				Acid	150	2,000		
B	7	Urine	500	Enzyme	130	120	8%	6%
				Control	113	26		
				Acid	320	1,700		
B	8	Butanol Extract	320	Enzyme	26	45	38%	8%
				Control	7	8		
				Acid	57	540		
C	9	Urine	120	Enzyme	19	25	33%	5%
				Control	7	0		
				Acid	43	550		
C	9	Butanol Extract	120	Enzyme	23	100	21%	10%
				Acid	110	950		
C	9	Butanol Extract	160	Enzyme	35	160	100%	89%
				Acid	35	180		
D	8	Butanol Extract	1,000	Enzyme	45	52	84%	43%
				Control	2.5	0		
				Acid	50	120		

by the enzyme digestion varies from 8 per cent to 100 per cent for the estrone-estradiol fraction while that portion of the estriol fraction hydrolyzed by the enzyme varied from 5 per cent to 89 per cent. In general, a greater proportion of the estrone-estradiol fraction, than of the estriol fraction, is conjugated with sulfuric acid. Presumably, the balance of the estrogen in all cases is conjugated with glucuronic acid.

DISCUSSION

The conjugation of estrogens in human urine, with both sulfuric and glucuronic acid, has been postulated (Cohen and Marrian, 1936) but no sulfate-conjugated estrogen has ever been isolated. This, no doubt, is due to the small amount of sulfate-conjugated estrogens present in human urine. However, this limitation is made less insurmountable with the development of fluorimetric analytical methods which permit quantitative estimation of as little as 0.5 micrograms of estrogen (Bates and Cohen, 1947; Jailer, 1947; Finkelstein et al., 1947). Utilizing such methods, together with specific enzyme hydrolysis, it is now possible to demonstrate the presence of such sulfate-conjugated estrogens in human urine. Of special interest is the finding that a large portion of the estriol in urine may sometimes be conjugated with sulfuric acid.

Since it has been shown that Mylase P contains no glucuronidase or general phenol esterase (Fishman, 1948) and since it also has been shown that Mylase P contains a phenolsulfatase (Abbott, 1947; Cohen and Bates, 1949) it is concluded that the urinary estrogens hydrolyzed by the enzyme mixture existed in the urine as a water-soluble sulfate. It may also be conjectured, that, inasmuch as the phenolsulfatase of aspergillus oryzae does not hydrolyze alcoholic sulfates (Fromageot, 1938), the point of conjugation of estriol with sulfuric acid must be at the 3 phenolic hydroxyl position in the estriol molecule, thus differing from the point of glucuronic acid conjugation, which has been shown by Marrian (1937) to be at the 16 or 17 alcoholic hydroxyl position.

SUMMARY

The occurrence of estrogens conjugated with sulfuric acid in pregnant women's urine was demonstrated by the use of an enzyme preparation containing phenolsulfatase.

The sulfate ester linkage must be with the phenolic hydroxyl group at position 3 since phenolsulfatase does not hydrolyze alcoholic sulfates.

Among seven different urine samples a variable proportion of from 5 per cent to 89 per cent of the estriol fraction and 8 per cent to 100 per cent of the estrone-estradiol fraction was found to be conjugated as the sulfate.

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PITUITARY AND ADRENO-CORTICAL RELATIONSHIPS TO LIVER REGENERATION AND NUCLEIC ACIDS

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WE HAVE found (1949) that thyroidectomy in young animals reduces the capacity for liver regeneration in rats after partial hepatectomy; and that administration of desiccated thyroid to both young (2 months old) and older (3 to 5 months old) animals increases the rate of regeneration. We here report experiments on the influence of the pituitary and the adrenals. The results indicate that hypophysectomy depresses liver regeneration, but that there is no evidence of any adrenal effect. Other results will be discussed in the text.

METHODS

Inbred rats of the Wistar strain (a few of the Sprague-Dawley) were used. Sex apparently had no influence on the results, and the results include data on both sexes. Partial hepatectomy was performed by removing the left lateral and median lobes. It has been amply established that this removes, for our purpose, with negligible error about 2/3 of the liver. The liver was allowed to regenerate for 96 hours, and the amount, which can be variously calculated, was determined by the following formula:

$$\% \text{ regeneration} = \frac{\text{wt. of liver at autopsy} - \frac{1}{2} \text{ amount removed}}{\text{amount removed}} \times 100.$$

This represents in per cent approximately the amount of removed liver replaced by new tissue.

All surviving animals were sacrificed 96 hours after partial hepatectomy, by which time the most rapid phase of regeneration has been completed. The animals were maintained on a standard diet of 25% casein, 50% sucrose, 20% Crisco, 5% salt mix (Phillips and Hart, 1935); and a vitamin supplement of Vipenta 0.5 ml/kilo. Hypophysectomy and adrenalectomy both affect the appetite; in both cases, therefore the normal animals were restricted to the average food intake of the group for which they acted as controls. The animals that were given adrenocortical extract were kept at the same dietary level as the adrenalectomized animals and their normal controls. The hypophysectomized animals and their controls were five weeks old at partial hepatectomy; the adrenal experimental group was two months old. Nucleic acids were determined by the method of Schmidt and Thannhauser (1945).

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RESULTS:

(1) *Pituitary.* The animals were hypophysectomized at age of 28 days, by Dr. Roy O. Greep, to whom we wish to express our obligation, and were lobectomized 5–7 days later. The data concerning this group and their controls are summarized in Table 1. The striking thing is the difference in the 96 hour regeneration. In the normal animals on a restricted diet this amounted to 76 per cent; in the hypophysectomized, 39 per cent. The "T" value was over 3, and the results statistically significant.

Franseen, Brues and Richards (1938) were of the opinion that the decreased liver regeneration of hypophysectomized animals was chiefly due to the diminished food intake, though Higgins and Ingle (1939) stated that "on the basis of total average animals, it is possible that the loss of pituitary gland may have had some inhibitory effect on the extent of regeneration." Their caution in interpretation in spite of the fact that the hypophysectomized rats actually did regenerate less than controls on the same diet, was due to doubts concerning the absorption and utilization of diets and differences in muscular activity. In so far as this might affect the relation of available nutritive material to the energetic needs, and hence the relative state of nutrition, this was not a factor in our experiments, since not only was the average amount eaten by the control and hypophysectomized animals the same, but their change in weight during the experiment was not significantly different. Unless the latter were coincidental, due to compensatory differences in hydration, which there is no reason to believe, we can say that the diet was not a factor in our experiments and that the influence of the pituitary on liver regeneration would appear to be established.

In the pre-lobectomized liver, hypophysectomy resulted in a fall in the ribonucleic acid (PNA) fraction, but no change in desoxyribonucleic acid (DNA), nitrogen, or water concentration. After four days' regeneration following lobectomy, there was what appeared to be a significant (though barely so) rise in the DNA concentration in hypophysectomized as compared with the control animals. This is of interest, because in the experiments cited above, we have found that thyroidectomy in young animals results not only in a decreased rate of liver regeneration, but also a fall in the nucleic acid concentration. It would seem therefore that the amount of nucleic acid mobilization and of regeneration do not necessarily go hand in hand. Also, so far as it goes, it tends to indicate that the pituitary influence on regeneration is not via the thyroid. This point, however, requires further investigation.

(2) *Adrenals.*

(a) *Adrenalectomy.* A group of animals were adrenalectomized and then partially hepatectomized 10 days later, when they were about two and one-half months old. At first, none of our adrenalectomized

TABLE 1. EFFECTS OF HYPOPHYSECTOMY

	Pre-lobectomized liver					Regenerated liver					
	Nucleic acids			Dry wt.	Total N	Nucleic acids			Dry wt.	Total N	Regeneration
	PNA	DNA	Total	Wet wt.		PNA	DNA	Total	Wet wt.		
	mgm. P/100 gm. wet wt.			%	%	mgm. P/100 gm. wet wt.			%	%	%
Normal (14)*	111±3.0†	25±2.4	138±3.8	30±0.4	3.24±.096	114±2.3	27±2.3	141±3.4	28±0.5	3.04±.055	76±6.5
Hypophysectomized (26, 10)	98±1.7	26±1.8	124±2.5	29±0.5	3.13±.082	115±2.8	38±2.7	153±3.9	28±1.4	3.23±.138	39±2.4

* Numbers in parentheses refer to number of experimental animals. Where there are two numbers in parentheses, the second indicates the number that survived hepatectomy for regeneration studies.

† Standard Deviation of the Mean = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

TABLE II. EFFECTS OF ADRENALECTOMY AND ADRENOCORTICAL EXTRACT INJECTIONS

	Pre-lobectomized liver					Regenerated liver					
	Nucleic acids			Dry wt.	Total N	Nucleic acids			Dry wt.	Total N	Regeneration
	PNA	DNA	Total	Wet wt.		PNA	DNA	Total	Wet wt.		
	mgm. P/100 gm. wet wt.			%	%	mgm. P/100 gm. wet wt.			%	%	%
Normal Kept at 20°–25° (10)*	102±0.9†	21±0.7	123±1.1	29±0.2	2.93±.081	110±2.8	22±0.4	132±2.8	30±0.6	2.55±.088	63±6.4
Normal Kept at 31° (12)	110±0.4	21±0.1	131±0.4	29±0.6	3.24±.042	111±0.6	23±0.6	134±0.8	30±0.7	3.23±.107	52±3.4
Adrenalectomized Kept at 20°–25° (16)	103±1.9	21±0.9	124±2.1	29±0.4	2.95±.046	123±2.3	28±1.0	151±2.5	26±0.3	2.68±.014	56±4.9
Adrenalectomized Kept at 31° (15)	108±1.5	26±0.6	134±1.6	36±0.3	3.74±.089	111±1.2	26±0.3	137±1.2	34±0.5	3.39±.116	46±5.0
ACE (Injected for 5 days) 5 rat units daily (10)	97±1.5	18±0.7	115±1.7	30±0.3	2.70±.024	119±3.4	21±0.4	140±3.4	29±0.6	2.55±.055	56±6.4
ACE (Injected for 5 days) 15 rat units daily (3)	104±2.1	15±0.0	119±2.1	30±0.0	2.96±.024	117±1.4	25±0.0	142±1.4	27±0.1	2.74±.170	49±6.5

* Numbers in parentheses refer to number of experiments.

† Standard Deviation of the mean = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

animals survived the removal of two-thirds of the liver, death occurring invariably within 24 hours. After some experimentation, however, we found that if 10 ml. of an isotonic NaCl solution was introduced into the peritoneal cavity at the time of hepatectomy, the great majority of the animals survived when given 1% NaCl in their drinking water. These were compared with a control group of the same age kept at the same food intake. The results are recorded in Table 2. They can be summarized by saying that adrenalectomy had no significant effect on the rate of regeneration, nor on the nitrogen or water content of the liver. There was, however, after adrenalectomy a greater concentration of nucleic acids in the regenerating liver than in the controls, apparently affecting both fractions. (The absolute amount was approximately the same). This is not observed in the liver before hepatectomy. Comparing this with the nucleic acid patterns described in the previous section, it is evident that no definite relationship exists between the regenerative activity of the liver under the different conditions considered, and the nucleic acid concentration.

Berman, Sylvester, Hay and Selye (1947) have recently reported that adrenalectomy decreases the amount of liver regeneration in the first 24 hours to an extent not accounted for by the decreased ability to mobilize fat. Our own results, for the 96 hour regeneration, as indicated, do not agree with this. Since the regeneration of the first 24 hours involves little or no mitosis, and is chiefly a matter of fat and water mobilization, possibly a distinction should be made between the regeneration of this period and that of later periods, since the liver events of the first day after lobectomy are in a sense largely a preparation for the cell regeneration that occurs later.

In view of the fact that a few adrenalectomized animals, in hot summer weather, showed a tendency to a lower regeneration than animals under more temperate conditions, we thought it might be possible that under such a stress the effect of adrenalectomy might be made manifest. Through the kindness of Dr. Eugene M. Landis, who made available to us a constant temperature room, we were enabled to maintain a group of adrenalectomized animals and their controls for ten days before, as well as for the four days after hepatectomy, at a temperature of 31°C. The results will be seen in Table 2. The adrenalectomized animals showed a smaller water content than their controls, and than both controls and adrenalectomized at moderate temperatures. Both groups at the high temperatures had a high nitrogen concentration, though little more than might be expected from the fall in water content. The regeneration was somewhat reduced in both groups. It is apparent that the stress of high temperature did not preferentially affect the adrenalectomized animals, except in the matter of the liver dehydration.

(b) *Injection of Adrenocortical Extract* (ACE). Into a group of ten animals we have injected five units of ACE[2] in two divided doses daily on the day prior to hepatectomy and during the four days following. It will be seen in Table 2 that this was entirely without effect. To three more animals we administered fifteen units daily in the same manner. This, too had no appreciable effect.

From this evidence, and that obtained from adrenalectomy, we must conclude that the adrenal cortex exerts no influence on the regeneration of liver. It follows, of course, that the demonstrated influence of the pituitary is not via the adrenal cortex.

SUMMARY

We have investigated the influence of the pituitary and adrenals on liver regeneration following partial hepatectomy, and upon nucleic acid and total nitrogen concentration, the effect of diet and nutritional status being controlled.

Hypophysectomy results in a marked diminution in regeneration and a change in nucleic acid partition and concentration.

Adrenalectomy causes an increase in nucleic acid concentration in the regenerated liver; this is not reversed when ACE is injected. However, neither adrenalectomy nor the administration of adrenal-cortex extract has any effect on the amount of liver regeneration.

The control exercised by the pituitary on the regeneration is hence not exerted through the adrenals; the evidence suggests that it is also not mediated through the thyroid.

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SEROLOGICAL STUDIES ON CRYSTALLINE ADRENOCORTICOTROPHIC HORMONE: THE PRODUCTION OF ANTI-ADRENOCORTICOTROPHIN[1]

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CURRENT interest in the adrenocorticotrophic hormone has continued to expand at a rapid rate, and a vast quantity of data concerning the physiology and metabolism of this hormone and its influence upon the adrenal gland, has been accumulated. In view of this widespread use of the adrenocorticotrophic hormone, both in clinical and animal experimentation, the problem of the formation of a substance antagonistic to the action of this hormone is a pertinent one.

It is a well known fact that certain hormones, when administered parenterally over a long period of time, stimulate the production of substances which have been called "antihormones." These substances have the property of inhibiting the action of any further injected hormone. The phenomenon of antihormone formation and action was discovered by Collip (1934; 1935) during the course of his work with the pituitary-trophic hormones; it was found that sera of refractory animals (those which did not respond to injections of pituitary gonadotrophic hormone) protected immature female rats against the normal action of this hormone. Collip and Anderson (1934) also demonstrated a similar result with the thyrotrophic hormone.

Although the mechanism of hormone-antihormone action is still a matter of controversy, the results of various investigators indicate that antihormones behave, for the most part, as antibodies; and that, therefore, the term *antihormone* may be defined immunologically as *an antibody produced in response to the antigen, hormone* (Thompson, 1946). The controversy, which is still in existence, is the result of the fact that relatively few of the hormones have been isolated in pure form, and of those which have been crystallized, few are available in large enough quantities for experimental purposes. As a result, those investigators who do not regard *antihormones* and *antibodies to*

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hormones to be synonymous, postulate that any *in vitro* antibodies which can be demonstrated in the sera of refractory animals are non-specific, in that they are due to impurities in the antigen preparations.

Of the pituitary hormones, the gonadotrophic, thyrotrophic and adrenocorticotrophic demonstrate the greatest degree of antigenicity (Thompson, 1946). The sera of normal, untreated individuals have been reported to contain antibodies to the gonadotrophic hormone, although this phenomemon occurs only in rare instances (Collip, 1935; and Collip and Anderson, 1939). Chow (1942) working with "pure" hog-pituitary gonadotrophic hormone, found this material to be antigenic, the homologous antiserum being both species- and hormone-specific. Equine gonadotrophic hormone (pregnant mares' serum), which is used extensively clinically, has also been shown to be antigenic (Rowlands and Spence, 1939; Jailer and Leathem, 1940). Allergic reactions have also been elicited with this preparation (Fluhmann, 1940; Erving, Sears and Rock, 1940; and Bickers, 1941).

Using 4 commercial preparations of gonadotrophic hormone, the author has previously demonstrated the formation of precipitins to each of these substances (Chase, 1945). These antibodies were shown to have identical properties with other immunologically defined antibodies. Suitable serological methods for demonstrating the precipitating antibodies were developed. These methods utilized collodion particles to increase the surface of the antigen, since the gonadotrophic hormone is thought to be a relatively small particle of glycoprotein. It was also shown in this series of experiments that the production of serologically demonstrable antibodies to the gonadotrophic hormone was accompanied by an increase in biological activity and *vice versa*. Electrophoretic studies in the Tiselius apparatus revealed an increase in the gamma-globulin portion of the blood serum of injected animals, which could be correlated with the increase in precipitating antibodies as well as with the increase in biological activity. The mechanically separated gamma-globulin exhibited three times the precipitating and biological activity of the whole serum. Since the nitrogen content of the gamma-globulin portion was shown to be much less than was that of the whole serum (Micro-Kjeldahl determinations), it was concluded that the antibody was contained in the gamma-globulin portion of the blood serum.

It has recently been shown that, in rabbits, the route of injection has a marked influence upon the rapidity with which inhibitory substances for the gonadotrophic hormone are formed (Leathem, 1947). Antihormones were formed more readily when the subcutaneous route of injection was used. These results have been confirmed by the author, using Gonadin, a commercial pregnant mares' serum preparation (Chase, unpublished results). In immunological circles, it is an

established fact that the antibody response to a given antigen is influenced markedly by the route of injection of the latter.

Again, there have been reports of the appearance spontaneously of the antithyrotrophic factor in the sera of untreated individuals (Simon, 1936; Picado and Rotter, 1936). However, it has been impossible to demonstrate biologically active antagonists in patients with Graves' disease until treatment has been instigated (Spence and Scowen, 1934; Picado and Rotter, 1936; and Scowen and Spence, 1936). The titer of anti-thyrotrophin has been reported to be high in a case of Cushing's syndrome (Eisenhardt and Thompson, 1939). Finally, reports of the development of antithyrotrophic factors in the sera of both man and animal do exist, and the results are similar to those obtained with the antigonadotrophic factor (Spence and Witts, 1939; Collip and Anderson, 1934; Okkels, 1937; Cope, 1938; and Cutting, Robson and Emerson, 1939).

The present study was undertaken to determine whether or not purified adrenocorticotrophic hormone would elicit antihormone formation after injection into suitable experimental animals. Further, in substantiation of the hypothesis that the reaction of a hormone and its specific antagonist is a true immunological phenomenon, it was sought to produce an *in vitro* reaction between the crystalline adrenocorticotrophic hormone and its homologous antiserum.

MATERIALS AND METHODS

Animals. Normal mice of both sexes (NHO strain, Strong; and Swiss white), 60–80 days of age, were used for the production of antibodies to the adrenocorticotrophic hormone. Twenty-one-day-old, white, male rats were used to study the biological activity of the antiserum against the adrenocorticotrophic hormone. Food and water were provided *ad libitum.* Both mice and rats were fed Purina Fox Chow. All animals were kept in the laboratory for at least one week prior to their use.

Hormone preparations. The adrenocorticotrophic hormone preparation used was the one described by Sayers, White and Long (1943). The preparation fulfilled two criteria for protein purity: it behaved as a single component in the Tiselius apparatus and in the ultracentrifuge. The preparation exhibited none of the following pituitary hormonal activities in biological assay determinations: growth, gonadotrophic, thyrotrophic or lactogenic.

Crude Prolactin was used as a control material in the biological assay experiments. From previous studies, this material was known to contain assayable quantities of adrenocorticotrophic hormone.

Antisera. Blood was obtained from all mice by cardiac puncture. The bloods were pooled, allowed to clot in the refrigerator and the sera separated by centrifugation. All sera were kept in the frozen state until needed.

In certain of the *in vivo* experiments, antigonadotrophic serum from a dog "Alice" was used (Thompson and Cushing, 1937). The dog was injected for many months with a crude sheep pituitary preparation, and its serum contained antagonists to the pituitary gonadotrophic hormone as well as to other pituitary hormones. The dog serum was maintained in the lyophilized state.

When needed, it was standardized so that 0.1 ml. of solution contained 5 mg. of lyophilized preparation.

Collodion particles. Collodion particles were used in several of the serological tests, to increase the surface of the antigen (adrenocorticotrophic hormone), and thus produce particles within the range of visibility. These were prepared according to the methods of Cannon and Marshall (1940) with a few modifications.

When the collodion particles were to be coated with antigen, a suspension dense enough to be milky, was added to about twice its volume of adrenocorticotrophic hormone, and the mixture was allowed to remain in the refrigerator over a period of 48 hours. If the particles settled while in contact with the antigen, they were readily resuspended by shaking. The coated particles were then washed by centrifugation and decantation and were finally resuspended in a small amount of triply distilled water. The pH was adjusted to 6.0 with 0.01N HCl and a buffer at this pH was added, in the proportions of one part buffer to 10 parts suspension. The buffer solution was prepared by mixing 12.5 ml. 0.2 M sodium acetate and 0.3 ml. of 0.2 M acetic acid and diluting to 250 ml. with triply distilled water. The buffer tended to stabilize the particles so that the adsorbed suspension could be kept in the refrigerator for longer periods of time.

When the adrenocorticotrophic hormone-collodion particle complex was needed as the antigen in the serological tests, a small quantity of the stock mixture was diluted with physiological saline until it corresponded with tube #5 of the MacFarland nephelometric standard. The antigen-collodion particle complex was then added in constant amounts to varying dilutions of the antiserum.

Protocol of immunization. The first group of mice received gradually increasing daily doses of pure adrenocorticotrophic hormone intraperitoneally over a period of 25 days. A total of 50 mg. of the hormone was administered to each mouse. Each dose of hormone was dissolved in 0.3 ml. of 0.85 per cent saline. Control mice received injections of saline alone. The mice were bled one week after the first injection, one day after the last injection and again, 10 days after the last injection.

The second group of mice were treated in exactly the same manner except that the injections were given subcutaneously. Ten control mice received injections of 0.3 ml. 0.85 per cent saline.

Biological Assay Methods. In studying the effect of pure adrenocorticotrophic hormone on the adrenal weights of 21-day-old, male rats, 0.05 mg. of hormone in 0.1 ml. 0.85 per cent saline were injected subcutaneously three times a day for three days. During this three-day period, the rats were weighed daily. Sixteen hours after the last injection, the rats were again weighed, then killed with illuminating gas. The adrenals were removed immediately and weighed on a torsion balance. The adrenal weights were finally expressed as mg./100 gm. initial body weight.

In the experiments where antiserum was injected, this was administered subcutaneously in 0.05 ml. quantities twice a day for 3 days so that a total of 3.0 ml. in all were injected.

When both the adrenocorticotrophic hormone and the antiserum were used, hormone injections were begun one day after serum injections and, therefore, lasted one day longer.

Serological Methods. When needed, the antiserum was allowed to thaw at room temperature. Progressive dilutions were added by the doubling dilution technique (1:2 to 1:2560) to a series of serological test tubes. All dilutions were made with 0.85 per cent saline prepared with triply distilled water, and each tube contained 0.5 ml. diluted serum. Each test was done in duplicate.

When adrenocorticotrophic hormone-collodion particle complex was used as the antigen, 0.5 ml. of a suspension corresponding to tube # 5 of the MacFarland nephelometric standard was added to each tube of diluted serum. Controls included 1:10 antiserum alone, 1:10 antiserum plus unadsorbed collodion particles, and hormone-collodion particle complex alone. Routinely the racks of tubes were incubated overnight in a water bath at 37 deg. C. After the incubation period, the tubes were centrifuged in an angle centrifuge at approximately 1200 r.p.m. for 5 minutes, and then "flipped." Tubes in which a precipitate occurred which adhered to the bottom of the tube and did not resuspend even after vigorous shaking, were interpreted as positive.

In several cases, precipitin tests were set up in which the adrenocorticotrophic hormone alone (unadsorbed) was used as antigen. Antiserum dilutions were made in the same manner as when the hormone-collodion particle complex was used as the antigen. To each tube of diluted antiserum, 0.5 ml. of 0.85 per cent saline containing 0.025 mg. adrenocorticotrophic hormone were added. The racks of tubes were incubated at room temperature for 2 hours. Tubes in which there occurred a macroscopically visible precipitate were recorded as positive.

EXPERIMENTAL AND RESULTS

Biological Assay Experiments. Since the adrenocorticotrophic hormone used in these experiments was a pure preparation, it was interesting to note whether or not a serum from mice, treated daily with this hormone for 25 days, would inhibit the normal action of the latter. The results of this experiment are recorded in Table 1. It may be seen from this table that the mouse anti-adrenocorticotrophic serum exerted a pronounced inhibitory effect on the normal action of the adrenocorticotrophic hormone. When a total of 0.45 mg. of hormone was injected into each of 5 rats, an average adrenal weight of 77.2 mg./100 gm. body weight was obtained. When both hormone and serum from mice, treated for one week with adrenocorticotrophic hormone, were injected into a group of 10 rats, the average adrenal weight was 51.3 mg./100 gm. body weight. With sera obtained 1 day after the series of injections had been completed, the inhibitory effect was even greater (average adrenal weight = 41.2 mg./100 gm. body weight). Sera obtained 10 days after the series of injections had been completed produced the most marked inhibitory effect on the normal action of the adrenocorticotrophic hormone (average adrenal weight = 36.3 mg./100 gm. body weight).

It may also be seen from the results in Table 1 that crude Prolac-

tin exerts a definite adrenocorticotrophic action, when injected into 21-day-old, male rats, in that the adrenal weights in these animals were greater than those of the normal controls.

When dog antigonadotrophic serum was used, the action of the adrenocorticotrophic hormone on the adrenals was inhibited although this inhibition was not as marked as when mouse antiserum was injected. The action of the crude Prolactin was also inhibited both when the dog antigonadotrophic serum and the specific mouse anti-

TABLE 1. BIOLOGICAL ACTIVITY OF MOUSE ANTISERA SPECIFIC FOR ADRENOCORTICOTROPHIC HORMONE

Number of rats	Treatment	Average adrenal wts./100 mg. body wt.
5	ACTH[1]	77.2 mg.
5	ACTH+Normal Mouse Serum	76.9 mg.
4	Crude Prolactin[2]	59.3 mg.
10	ACTH+Antiserum I[3]	51.3 mg.
6	ACTH+Antiserum II[4]	41.2 mg.
6	ACTH+Antiserum III[5]	36.3 mg.
6	Crude Prolactin+Antiserum III	44.0 mg.
6	Crude Prolactin+dog (Alice) antigonadotrophic Serum[6]	50.9 mg.
8	ACTH+dog (Alice) Antigonadotrophic Serum	41.6 mg.
6	Pooled normal Mouse Sera	35.5 mg.
6	—	35.1 mg.
6	Antiserum III	27.5 mg.
6	Dog (Alice) Antigonadotrophic Serum	34.6 mg.

[1] Pure adrenocorticotrophic hormone. A total of 0.45 mg. in 0.9 ml. 0.85 per cent saline was injected, intraperitoneally.
[2] A total of 9 mg. in 0.9 ml. saline was injected intraperitoneally.
[3] Antiserum I was obtained from mice one week after the first injection of ACTH.
[4] Antiserum II was obtained one day after the series of injections had been completed.
[5] Antiserum III was obtained 10 days after the series of injections had been completed.
[6] A total of 30 mg. in 6.0 ml. saline was injected subcutaneously.

adrenocorticotrophic serum were used; and again the inhibitory effect was greater with the latter.

Control animals included one group in which no injections were given, and a second in which normal mouse serum was used. Results were comparable in both control groups. When the specific mouse antiserum was injected without the stimulating hormone, the adrenal weights were slightly less (27.5 mg./100 gm. body weight) than those of the control groups (35.5 mg./100 gm. body weight; and 35.1 mg./100 gm. body weight), although the difference was not great enough to draw any conclusions as to the effect of the antiserum on the intact adrenal.

To all intents and purposes, the results of this experiment indicate that it is possible to produce in mice a serum which exerts a profound antagonism against the normal action of the adrenocorticotrophic hormone in its pure state. The titer of antagonistic factor in-

creases during the course of immunization and reaches a peak 10 days after the series of injections has been completed. Serum obtained at this time, and injected into the 21-day-old, male rats has been shown to inhibit completely the effect of the adrenocorticotrophic hormone in increasing adrenal weight.

Serological Experiments. As has been previously stated, definite proof of the nature of antihormones has not yet been offered because a pure protein hormonal antigen has never been used for the production of these antagonistic substances. Current opinion, however, favors the theory that antihormones belong to the class of substances called antibodies (Thompson, 1946). The results of the above *in vivo* experiments indicate that it is possible to produce in animals a substance, present in the serum, which has the biological effect of

TABLE 2. AGGLUTININ TITERS OF ADRENOCORTICOTROPHIC HORMONE-COLLODION PARTICLE COMPLEX AND POOLED MOUSE ANTI-ADRENOCORTICOTROPHIC SERA

Number of mice	Treatment	Route of injection	Agglutinin titers, serum obtained			
			Pre-injection	1 week after 1st injection	1 day after last injection	10 days after last injection
20	ACTH[1]	Intraperitoneal	0	1:20	1:80	1:320
10	Controls—0.3 ml. 0.85% saline daily	Intraperitoneal	0	0	0	0
20	ACTH	Subcutaneous	0	1:40	1:160	1:320
10	Controls—0.3 ml. 0.85% saline daily	Subcutaneous	0	0	0	0

[1] Adrenocorticotrophic hormone. Each mouse received a total of 50 mg. in gradually increasing daily doses over a period of 25 days.

neutralizing the normal action of the pituitary adrenocorticotrophic hormone. Since the hormone used in these studies has been shown to be a single protein, it may be assumed that the antiserum is directed against the hormonal antigen and not against any contaminating protein antigen. Although the inhibition experiments may be likened to the passive immunization studies, so well known to all immunologists, it was sought to produce an *in vitro* reaction between the antigen, adrenocorticotrophic hormone, and the antibody, anti-adrenocorticotrophic factor, contained in the sera of mice which had received a prolonged course of injection of the antigen.

Two varieties of serological tests were employed although, in essence, their nature was identical. The first utilized collodion particles upon which the hormonal antigen had been adsorbed. Since the antigen is normally a soluble one, this process produced a large particle

which, when aggregated, was macroscopically visible. This type of test will hereafter be referred to as an *agglutination* test.

The results of the agglutination test are summarized in Table 2. It may clearly be seen from the results that, in the test tube, a macroscopically visible reaction occurs between the adrenocorticotrophic hormone-collodion particle complex and the specific mouse antiserum. Serum obtained before the course of injections had been initiated, failed to produce this reaction. The maximum titer (1:320) was obtained with the serum taken 10 days after the series of injections had been completed. Antibodies to the adrenocorticotrophic hormone were formed more readily and more rapidly when the subcutaneous route of administration was used. These results are in accord with those obtained in the *in vivo* experiments.

TABLE 3. PRECIPITIN TITERS OF ADRENOCORTICOTROPHIC HORMONE AND HOMOLOGOUS MOUSE ANTISERA

Number of mice	Treatment	Route of injection	Precipitin titers, serum obtained			
			Pre-injection	1 week after 1st injection	1 day after last injection	10 days after last injection
20	ACTH[1]	Intraperitoneal	0	1:20	1:80	1:320
10	Controls 0.3 ml. 0.85% saline	Intraperitoneal	0	0	0	0
20	ACTH	Subcutaneous	0	1:40	1:160	1:640
10	Controls 0.3 ml. 0.85% saline	Subcutaneous	0	0	0	0

[1] Adrenocorticotrophic hormone. Each mouse received a total of 50 mg. in gradually increasing daily doses over a period of 25 days.

Although collodion particles are considered to be an inert material, the possibility exists that they may exert some influence on the reaction between the adrenocorticotrophic hormone and its homologous antiserum. Therefore, a precipitin test was set up in which the antigen was the soluble hormonal protein. The results of this experiment are shown in Table 3. The maximum titer in the precipitin test was 1:640. This titer occurred with the serum obtained 10 days after the series of injections had been completed. As was the case with the agglutination test, precipitating antibodies appeared more rapidly when the subcutaneous route of injection was used in the production of the antisera.

Finally, an experiment was devised in which the supernatant layers from tubes containing visible precipitates were tested for biological activity. The supernatant layer was carefully removed from each tube after centrifugation, and was injected intraperitoneally into 21-

day-old, male rats. Each rat received 0.05 ml. 3 times a day for 3 days. Sixteen hours after the last injection, the rats were killed with illuminating gas, and their adrenals removed and weighed on a torsion balance. The results are tabulated in Table 4.

The results of this experiment confirm the results of the biological assay experiments. When normal mouse serum is combined with 0.025 mg. of adrenocorticotrophic hormone, no visible serological reaction occurs. Since there is no neutralization of the hormone action, approximately a twofold increase in adrenal weight is produced when

TABLE 4. EFFECT OF SUPERNATANT LAYERS FROM PRECIPITIN TESTS ON THE ADRENAL WEIGHTS OF 21-DAY-OLD, MALE RATS

Supernate Injected	Adrenal wt./100 gm. body wt. Rat injected with dilution								
	1:2	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
0.025 mg. ACTH[1] + Pooled Mouse Sera (Pre-injection)	61.2	60.0	58.4	60.9	59.8	61.0	62.4	61.1	61.8
0.024 mg. ACTH + Pooled Mouse Sera Obtained one Week after 1st Injection	29.5	31.0	30.8	31.5	29.9	36.4	47.0	54.3	59.8
0.025 mg. ACTH + Pooled Mouse Sera Obtained one Day after Last Injection	27.4	28.0	29.2	27.6	29.9	28.5	30.1	35.9	41.4
0.025 mg. ACTH + Pooled Mouse Sera Obtained 10 Days after course of Injections had been Completed	23.9	25.2	27.8	26.2	24.9	28.0	29.7	28.8	30.6
Control—Injected with Pooled Mouse Sera Obtained 10 Days After Completion of Series of Injections	20.9	20.0	22.3	22.0	25.7	25.4	28.6	28.0	27.2
Control—Saline	32.4	35.7	33.9	—	—	—	—	—	—

[1] Pure adrenocorticotrophic hormone.

the mixture is injected intraperitoneally into 21-day-old, male rats. However, when pooled mouse sera, obtained one week after the first injection of adrenocorticotrophic hormone had been given, was titrated against the hormone in the precipitin test, a titer of 1:40 was obtained. After the precipitate had been removed by centrifugation, the supernates of the 1:2, 1:10, 1:20, 1:40, 1:80 and 1:160 dilutions, produced no increase in adrenal weights of rats injected with the varying serum dilutions. In the dilutions beyond 1:160, the effect of the adrenocorticotrophic hormone was elicited. Sera obtained after the series of injections had been completed, produced comparable results both in the serological tests and in the biological assay experiments, in which the supernates from the precipitin tests were used.

It is interesting to note that, when varying dilutions of serum obtained 10 days after the series of injections of adrenocorticotrophic hormone had been completed, were injected into 21-day-old, male rats, there was approximately a 30 per cent decrease in adrenal weights over the saline controls. The results recorded in Table 1 suggested that the specific antiserum for the adrenocorticotrophic hormone may exert a direct influence on the intact adrenal glands of the 21-day-old, male rat. The results of the present experiment confirm these suggestions.

DISCUSSION

The data on the production of anti-adrenocorticotrophic sera is scarce. Thompson and Cushing (1934) mention the occurrence of refractoriness to the adrenocorticotrophic hormone in animals. These workers attempted to reproduce the Cushing syndrome in a puppy by injecting large doses of crude, ovine pituitary extract over a period of 4 months. The attempt failed, the researchers attributing their lack of success to the formation of anti-adrenocorticotrophic factor, after constant and prolonged injection of the pituitary extracts. Since the extracts used in these experiments were crude, however, no definite conclusions could be drawn as to the formation and nature of the antiserum.

The data presented in the present report substantiate previous suggestions of anti-adrenocorticotrophic formation after continuous parenteral administration of the protein hormone. The sera of mice, injected with pure adrenocorticotrophic hormone over a period of 25 days, gradually developed the ability to neutralize the action of the latter upon the intact adrenals of 21-day-old, male rats. A maximum titer of inhibition was obtained with sera taken 10 days after the course of injections of adrenocorticotrophic hormone had been completed.

Since the preparation of adrenocorticotrophic hormone used in these experiments was a pure protein, as shown electrophoretically and in the ultracentrifuge, it was possible to investigate the nature of the reaction between a hormone and its homologous antihormone. The results of serological studies, using the pure adrenocorticotrophic hormone as antigen, and pooled sera of mice, injected daily with this hormone over a period of 25 days, as a source of antibody, demonstrate conclusively that the reaction of the adrenocorticotrophic hormone and its respective antihormone is an immunological phenomenon and may be likened to any other reaction between an antigen and its homologous antibody. It is interesting to note that a prozone phenomenon occurred regularly in the precipitin test. Since this immunological phenomenon occurs commonly with many immunologically defined antigens and their homologous antisera, its occurrence with the hormonal antigen and the anti-adrenocorticotrophic sera may be interpreted as an indication of the immunological nature of the reaction between a hormone and its respective antihormone.

In the final experiment of this series, an attempt was made to correlate the biological activity of the specific antiserum, obtained by continuous injection of adrenocorticotrophic hormone into mice, with the serological activity. For this purpose, the precipitates formed in the test tube were removed by centrifugation, and the supernatant layers were tested for their biological activity. There was a definite correlation between biological and serological activities, as shown

from the results of this experiment. In serum dilutions, where proportions of antigen (adrenocorticotrophic hormone) and antibody (pooled mouse antisera) were optimal, the supernates showed no demonstrable effect on the adrenal weights of 21-day-old, male rats. When an excess of antigen was present in the supernate, there were increases in the adrenal weight of rats proportional to the quantity of hormone present. Under opposite conditions (when an excess of antibody was present), the size of the intact adrenals of 21-day-old rats was decreased proportionally.

The results obtained in this experiment indicate that the precipitate, formed in the serological tests, consists of a combination of hormone and antihormone and that, under conditions of optimal proportions, the precipitate represented the neutralized complex. As has been stated, the supernatant layers, in such cases, showed neither hormonal nor antihormonal activity when used in the biological assay experiments.

The experimental evidence presented in this paper offers data which support any previous hypotheses that antagonistic substances are formed in the sera of animals treated continuously with adrenocorticotrophic hormone. Since the hormone used was a pure one, it may be concluded that the antagonistic substance must be specific for the adrenocorticotrophic hormone, and not the result of stimulation by an unrelated contaminating adjunct. Further, the identity of the hormone-antihormone reaction with a true immunological phenomenon, in which a reaction takes place between an antigen and its homologous antibody, has been established. The serological activity of the antibody specific for the adrenocorticotrophic hormone has been correlated with the biological activity.

SUMMARY

A substance antagonistic to the normal action of the adrenocorticotrophic hormone has been demonstrated in the sera of mice treated by increasing daily doses of pure adrenocorticotrophic hormone over a period of 25 days. These sera were titrated in biological assay experiments against standard doses of the hormone in question, and were shown to prevent the increase in adrenal weights normally brought about by the adrenocorticotrophic hormone.

The serological activity of the antisera was demonstrated in both agglutination tests, in which collodion particles were used to increase the surface of the hormonal antigen, and in precipitin tests. Pure adrenocorticotrophic hormone was used as the antigen and the above described sera as the source of antibody. Maximum serological activity was obtained 10 days after the course of injections had been completed. The serological activity of the antiserum could be correlated with its biological activity.

ACKNOWLEDGMENT

The author is indebted to Dr. Abraham White for making the various hormone preparations available, and for his invaluable suggestions during the course of these experiments.

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NOTES AND COMMENTS

THE LEVEL OF PLASMA PSEUDO-CHOLINESTERASE IN HYPOPHYSECTOMIZED AND HYPOPHYSECTOMIZED THYROXINE-TREATED RATS[1]

It has been shown previously (Hawkins, Mendel and Nishikawara, 1948; Hawkins, Nishikawara and Mendel, 1948) that a depression in the level of thyroxine induced by thyroidectomy or by treatment with thiouracil results in a highly significant elevation in the activity of pseudo-cholinesterase in the plasma of male rats and, conversely, that an elevation in the level of thyroxine leads to a depression in the activity of this enzyme which is also highly significant. The level of true cholinesterase, on the other hand, is not affected by any of the above procedures.

This investigation has now been extended to include an evaluation of the effect of hypophysectomy on the inverse relationship between thyroxine level and plasma pseudo-cholinesterase activity in order to determine whether the hypophysis is an essential link in the reaction whereby the above relationship is established. The results of these experiments are to be reported here.

EXPERIMENTAL PROCEDURE

Adult male Wistar rats (200–250 g.) were divided into three groups, the members of two of which were hypophysectomized by the parapharyngeal approach. Functional hypophysectomy in all cases was established by histological evidence of adrenal cortical atrophy when the animals were sacrificed.[2] The rats were maintained on a moist diet of bread, milk and ground meat, with a water ration containing 10% sucrose. They were given daily injections of glucose and were housed in a constant temperature room at 28°C throughout the experimental period. Starting three weeks after operation, the survivors in the third group received injections of thyroxine[3] (0.25 mg./100 g. body weight) every second day for a period of two weeks. Five weeks after the initiation of the experiment all animals were killed by exsanguination through the carotid artery under light ether anaesthesia. Oxalated blood samples were obtained and the plasma was used for estimation of pseudo-cholinesterase activity.

Enzymatic determinations were carried out manometrically in a medium of 0.025M $NaHCO_3$ saturated with 5% Co_2 (pH 7.4) with benzoylcholine (0.006M) serving as substrate (Mendel, Mundell and Rudney, 1943).

RESULTS

As the results in the accompanying table show, the pseudo-cholinesterase level in the plasma of hypophysectomized rats is elevated significantly above

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[1] Supported by grants from the Banting Research Foundation and Hoffman-La Roche Co. Ltd., Canada.

[2] We are indebted to Dr. W. S. Hartroft of this department for the histological examination of the adrenal glands.

[3] Roche Organon (Synthetic Thyroxine).

TABLE 1. THE EFFECT OF THYROXINE ADMINISTRATION ON THE LEVEL OF PSEUDO-CHOLINESTERASE IN THE PLASMA OF HYPOPHYSECTOMIZED MALE RATS*

Group	No. of animals	Plasma Pseudo-ChE Activity	t	P
Normal	10	26.6 ± 2.7†	—	— —
Hypophysectomized	6	51.3 ± 6.6	4.1‡	1.9×10^{-4}
Hypophysectomized, thyroxine-treated	8	15.7 ± 3.4	5.9§	3.1×10^{-7}

* Activity is expressed as cu. mm. CO_2 evolved in 15 min. by 1 ml. plasma.
† ± represents standard deviation of the mean.
‡ 't' and 'P' values are in relation to values obtained for normal animals.
§ 't' and 'P' values are in relation to values obtained for hypophysectomized animals.

that found in unoperated control animals. A comparison of the enzyme levels found in the hypophysectomized, thyroxine-treated group with those observed in the hypophysectomized animals reveals that thyroxine is still capable of exerting a depressing action on the level of plasma pseudo-cholinesterase, in spite of the absence of the hypophysis. The hypophysis is therefore not an essential link in the reaction whereby the inverse relationship between the level of thyroid function and plasma pseudo-cholinesterase activity is established.

SUMMARY

Hypophysectomy leads to an elevation in the level of plasma pseudo-cholinesterase in male rats. This elevation is statistically significant.

Treatment of hypophysectomized rats with thyroxine results in a significant depression in the level of pseudo-cholinesterase in the plasma.

The hypophysis is therefore not an essential link in the reaction whereby the inverse relationship between the level of thyroid function and plasma pseudo-cholinesterase activity is established.

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ASSOCIATION NOTICE

THE SCHERING FELLOWSHIP FOR 1949 IS GIVEN TO DOCTOR D. LAWRENCE WILSON

Doctor D. Lawrence Wilson was selected as the first recipient of the Schering Fellowship. Doctor Wilson will work under the direction of Doctor George Thorn in the field of metabolic and endocrine diseases.

Doctor Wilson received the degree of Doctor of Medicine at Queen's University in 1944. Following his internship he served for eighteen months as Regimental Medical Officer in the R.C.A.M.C. He then received a Medical Research Fellowship for two years from the National Research Council of Canada, working during this period in the Department of Biochemistry of the University of Toronto. He fulfilled as Research Fellow the requirements for the degree of Master of Arts which he received in June 1948.

THE SQUIBB AWARD FOR 1949 IS GIVEN TO DOCTOR HERBERT M. EVANS

About thirty years ago Professor Herbert M. Evans began his studies on the Physiology of Reproduction. Some of this earlier work was published with J. A. Long in a monograph, "Oestrous Cycle of the Rat," a contribution which was of great importance in the investigation and subsequent isolation of one of the ovarian hormones. During the following three decades many significant contributions to Endocrinology were made by Evans and his associates, the most important perhaps being the discovery of the growth hormone and its subsequent purification and isolation.

Other important aspects of endocrinology have been studied by Evans and his associates in the University of California: (1) the corticotrophic hormones of the hypophysis; (2) the gonadotrophic hormones of the hypophysis, pregnant mare serum, and of the chorion; (3) the impairment of the vaginal cycle due to Vitamin A deficiency; (4) the recognition of the importance in the rat of a nutritional factor, X, for pregnancy and subsequently, the isolation and characterization of this factor, Vitamin E.

Doctor Evans was born in California in 1882. He studied medicine at Johns Hopkins, receiving the M.D. degree in 1908. He remained on the faculty of that institution until 1915 when he was called to the Chair of Anatomy at the University of California. In 1930 he was made Herzstein Professor of Biology and Director of the Institute of Experimental Biology.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP FOR 1949 IS GIVEN TO DOCTOR ERNEST M. BROWN, JR.

Doctor Ernest M. Brown, Jr. was named to receive the Ayerst, McKenna and Harrison Fellowship for 1949. He was born in 1919 and received the degrees of Bachelor of Arts from West Virginia University in 1941 and of Doctor of Medicine from the University of Pennsylvania in 1944. He served

as intern and junior resident at the University of Pennsylvania Hospital 1944–46. Since that time until April 1948 he was a member of the Army Medical Corps. Doctor Brown will work at the George S. Cox Medical Research Institute with Doctor F. D. W. Lukens on lesions of the islands of Langerhans produced by intravascular infusion of glucose, continuing his studies begun under this Fellowship in 1948.

THE CIBA AWARD FOR 1949 IS GIVEN TO DOCTOR GEORGE SAYERS

Doctor Sayers developed a new and sensitive method for the assay of the adrenocorticotrophic hormone of the anterior pituitary gland. He found that the ascorbic acid and cholesterol content of the adrenal glands varies inversely with the amount of ACTH administered to the test animal. Having established the method, he applied it to the problem of pituitary: adrenal relationships. The interrelation of the pituitary and the adrenal cortex, and the response of this hormonal system to a variety of stimuli are better understood and can be better studied as a result of his investigations.

Doctor Sayers was born in 1914. He received the degree of M.S. in physics from the University of Michigan in 1936 and the Ph.D. degree in physiological chemistry from Yale University in 1943. From 1943 to 1945 he served with the Office of Scientific Research and Development at Yale University and in 1945 became Assistant Professor of Pharmacology at the University of Utah.

ERRATA NOTICE

In the paper by Hansen, entitled "A Modified Pettenkofer Reaction for the Quantitative Estimation of Dehydroisoandrosterone and Its Application to Analysis of Urinary Extracts and Fractionations" which appeared in Endocrinology 44: 492 (June) 1949, the following error has been noted:

On page 492, footnote 1, should read:

[1] The author is indebted to Dr. Wm. H. Stoner of the Schering Corporation, Bloomfield, N.J., and to Dr. E. Oppenheimer of the Ciba Corporation, Summit, N.J. for the steroids, to Dr. W. H. Pearlman of Jefferson Medical College for the bile acids, and to Dr. A. Rakoff of Jefferson Medical College for the clinical material employed in this investigation.